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Journal of DENTAL RESEARCH

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EDITOR'S VIEWPOINT

Rickles (p. 3) evaluated the Fosdick caries activity test, the L.a. index, and a new test based on the pH of incubated saliva-sucrose mixtures. All three tests correlated well with the increment of new carious lesions during the year following the tests, but the new colorimetric test is suggested as the "most accurate, simple, and effective" for routine office practice. If prediction or susceptibility tests for caries are to become more than research tools and be generally used in dental practice, they must be conducted easily by the dentist with his usual bacteriologic and chemical training.

Backer Dirks, Winkler, and van Aken (p. 18) used a precise method which they described previously (*J. D. Res.* 30: 346, 1951) to evaluate the effectiveness of a 5 per cent ammonium phosphate, 3 per cent urea dentifrice in controlling caries. They found it valueless. The approach they used in examination and evaluation is worthy of most careful consideration.

The search for the cause of caries reduction by fluorides led Clapper, Downs, and Heatherman (p. 27) to investigate the fermentative types of lactobacilli in the mouths of children from fluoride-free and fluoride-positive water regions. While no clear-cut type variation was found, it suggested that the type of lactobacillus may be related to caries activity only in the absence of fluorides, or that when fluorides are present large numbers of "active type" lactobacilli may be necessary to increase caries activity. More of this basic type study in caries-immune and caries-susceptibles is needed in fluoride-free as well as fluoride-controlled areas.

Bruce and Gunter (p. 35) investigated the caries and mottled enamel rates among a group of children who had been exposed to natural fluorides in the water periodically. They noticed beneficial effects on fluoridation of the teeth even when the water was intermittently fluoridated in ranges from 0.2 to 1.0 ppm. The correlation of between chronic endemic dental fluorosis, dental caries, and the degree of fluoridation of the water are discussed.

Burnett and Scherp (p. 46) have interested themselves in the organisms that may participate in dentin destruction during the process of dental caries. They found that lactobacilli were strong dentin decalcifiers, actinomycetes very moderate in their action, and cocci weak. Similarly lactobacilli made dentin matrix accessible for the action of lytic-enzymes followed in decreasing order by actinomycetes, proteolytic bacteria, and cocci. The actinomycetes produced discoloration similar to that seen in the base of long-standing cavities. It appears that the carious process in dentin is complex and that no single type of organism completes the many chemical reactions required.

Because three amino acids have been recognized in the salivas of human beings, and because it has been reported that the ingestion of relatively large doses of single amino acids results in changes in the blood amino nitrogen level, Kirsch, Kesel, O'Donnell, and Wach (p. 57) undertook an investigation of the amino acid levels in the human saliva. It was found that the amino acid level of twelve amino acids rose when the corresponding amino acid was given normally, but after thirty to sixty minutes the values returned to normal.

Drugs which are antibacterial and are retained for long periods on epithelial surfaces should be worthy of investigation as anticaries agents. The diphenylmethane derivatives have been shown to reduce total skin flora over prolonged periods and are presently employed in hand soaps. Ennever, Robinson, and Kitchin (p. 61) found three of these agents very effective against lactobacilli, one of them, G-11, being especially promising.

The flora of the dentobacterial plaque, as indicated by the study of Dreizen and Spies (p. 65) and others, has definite nutritional requirements. These bacterial nutritives may be highly significant in caries-susceptibility and immunity. As we learn more of the general growth requirements of the bacterial inhabitants of the mouth, and extend this to specific requirements of particular elements of the oral flora, we may open new avenues to caries control.

Ockerse (p. 74) has introduced a new animal into the caries study group. The African white-tailed rat appears to be caries

resistant unless its mother is fed a high sugar diet. This prenatal and lactation effect has been reported in other species, but how the conditioning effect produces change has not been explained.

Bender, Pressman, and Tashman (p. 78) found that parenterally injected penicillin or streptomycin had no significant effect on the total salivary bacterial count, while chloramphenicol an a penicillin-streptomycin mixture did reduce the oral bacterial population. Because of these data, it is proposed that penicillin-streptomycin mixtures be administered to prevent bacteremias and their sequelae after extraction. Of course, such procedure would not be routine but used only where systemic and local conditions warrant.

Fosdick and Piez (p. 87), continuing the investigation of periodontal disease by a study of chemical relationships, have isolated a number of free amino acids in salivas of patients with and without periodontitis. However, the salivas from patients with periodontitis contain larger amounts of unidentified volatile amines and a substance believed to be δ-aminovaleric acid. The suggestion is made that putrefaction takes place in the mouth only when adequate substrate (exudate and destroyed tissues found in periodontal pockets) is present before the putrefaction process. This study of periodontal disease offers another valuable approach and, in part, a reply to the many critics who suggest that no investigations are being made in the field of periodontal disease.

Fleming (p. 101), extending his study of ocular transplants of tooth germs, used cortisone as a possible deterrent to lymphocytic infiltration. Although cortisone-treated animals showed early vascularization of transplants, which grew rapidly at first, the transplants in untreated animals quickly surpassed those in cortisone-treated animals after the untreated transplants became vascularized. The use of hormonal fractions must be carried out with caution for many of their apparent beneficial results may shortly be overcome by detrimental changes following loss of enzymatic balance.

Yamada, Maruhashi, Kyozo, Hara, and Masuda (p. 110) have approached the problem of ennervation of the tooth from the standpoint of electrical impulses. This problem, which has interested the dental histologist and dental physiologist for some time, still is far from finally settled. However, it should be pointed out that the present study opens the way for further investigation of the response of the dentino-enamel junction, of predentin and the odontoblast, as well as other areas of the dentin. Again, the results offered strongly suggest that nerve fibers do not terminate within the dentinal tubules.

Dale's investigation of the effect of total body irradiation on the teeth of rats (p. 117) shows that the ameloblastic layer is particularly sensitive to radiant energy. This sensitivity of the functioning ameloblasts has been repeatedly demonstrated with chemical, bacterial, and traumatic injuries, and places the enamel in a peculiar position as a recording device for mild and severe injuries to the body during tooth development.

The mixed saliva obtained from the mouth has been investigated, chemically, more frequently than the saliva as a pure product of salivary glands. Burstone (p. 126) applied histochemical techniques to investigate the salivary glands in the mouse tongue. Observations indicate differences in the state of organization of the glycoproteins of the mucous and serous glands. The possible changes in the histochemical reactions of these glands under effects of aging, nutritional alterations, radiation, etc., seem worthy of further investigation.

Butcher (p. 133) has demonstrated pigment particles, similar to those seen in rat hair, which form in the ameloblast and apparently penetrate into the matured or maturing enamel of the rat. Fluorine leads to a late formation of pigment. As is pointed out by Pindborg and others (*J. D. Res.* **31**: 805, 812, 815, 1952), other materials may interfere with pigment production or deposition.

Russell (p. 138) studied caries data from 183 adults from Colorado Springs and 68 from Boulder. No correlation could be shown between the basic caries-susceptibility of approximal areas and caries inhibition area by area. There is a regular diminution of caries inhibition from the anterior to the posterior of the mouth and greater protection of mandibular approximal surfaces. Russell compares these data with the theoretical concepts of Besic, Nevin, and Walsh and Suess and Fosdick.

H. B. G. R.

THE ESTIMATION OF DENTAL CARIES ACTIVITY BY A NEW COLORIMETRIC LABORATORY TEST

A Preliminary Investigation

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SEVERAL laboratory procedures have been described in the past for the determination of dental caries activity; perhaps the most widely employed of these is the *Lactobacillus acidophilus* (L.a.) index. Many investigators have shown that this index varies proportionately, either with the number of carious lesions and restorations present at the time of examination, or with the dental caries increment.[1, 2, 4, 7, 12, 13, 15, 18, 19, 22] This laboratory has demonstrated in large scale clinical investigations[1, 2, 3, 6] that patients with rampant dental caries exhibited high L.a. indices, and that the remission of the carious process could be attained by a restricted refined carbohydrate diet, accompanied by drastic falls in the L.a. indices.

Fosdick, Hansen, and Epple[9] developed a chemical test for assessing dental caries activity, by measuring the increase in calcium of a saliva-glucose-enamel mixture after four hours' incubation at 37° C. Dewar[7] modified this test by determining, at the end of four hours' incubation, the pH of the saliva-glucose-enamel mixture. Standards were set up which gave a definite correlation between the test and the DMF teeth. Good or fair relationships occurred in 85 per cent of the cases. Both Fosdick's and Dewar's tests take into consideration the acidogenic potential of the "total" oral flora as does the colorimetric test for estimation of dental caries activity as proposed by Snyder.[17, 18] This approach appears more appropriate in the evaluation of dental caries activity than does the estimation of the numbers of any specific acid-producing organism in the oral cavity. Dreizen, Mann, Cline, and Spies[8] measured the buffering capacity of saliva of fifty patients and found that their laboratory results correlated well with the clinical dental findings.

Wach, Kesel, Hine, and O'Donnell[25] tested the dental caries activity of fifty patients over a two-year period (818 tests) by incubating 3 c.c. of each subject's resting saliva with 0.4 c.c. of 1 per cent glucose, and then determining the pH and total acidity at the end of four hours. In 86 per cent of the test results there was an indication as to whether caries would occur or not; in 22 per cent a good correlation between the pH and new caries existed; 64 per cent of the cases showed a moderate correlation, and in 14 per cent no correlation existed. Unfortunately, the method for determining the pH at the end of the four hours was not specified. Furthermore, it may be questionable whether sufficient glucose was supplied for saliva samples in which large numbers of rapid acid-producing microorganisms are present. However, except for the

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method of pH determination, the technic described is one which can conveniently be adapted for use in the dental office because it is a reasonably accurate, simple, and inexpensive test, and can be performed by nonprofessional personnel.

Although these methods are helpful, none of them seem to meet the needs of routine dental practice for one or more of the following reasons: (1) They require highly specialized or expensive equipment, (2) specifically trained technicians are essential, (3) the tests take too long to complete, (4) there is difficulty in discerning color changes, or (5) they demand excessive laboratory space and time for their analyses. Since these procedures have been used to predict only whether dental caries would occur or not, it may be well to consider the use of statistical manipulation of test results of a new procedure in order to predict more accurately the probable number of new carious lesions during the subsequent year, and, on this basis, estimate individual dental caries activity.

It is the purpose of this preliminary investigation to evaluate: (1) The accuracy and practicability of a new colorimetric laboratory test for dental caries activity (as measured by the dental caries increment), (2) the degree of correlation of total titratable acid (of an incubated saliva-sucrose mixture) with the increment in dental caries, (3) the degree of correlation of the L.a. index with the increment in dental caries, and (4) the relationships which may exist among the three tests.

MATERIAL AND PROCEDURE

Clinical and bite-wing roentgenographic dental examinations were made of 135 University of California students (ranging from 18 to 34 years, with a mean age of 22). The findings were recorded according to the modified Bodecker caries index.[5] Early morning paraffin-stimulated saliva samples were collected for single determinations of (these samples ranged from 48 to 96 hours in age):

1. The pH's of 134 of the 135 saliva samples, after being mixed and incubated with a sucrose-indicator solution.*

2. The total amount of acid produced after incubating these "saliva-sucrose" mixtures of 129 of the students for seven hours was found by titrating the washed contents of each sample tube with 0.02 N sodium hydroxide, using a Beckman pH meter set at pH 6.6 (the original pH of each test sample).

3. The L.a. index for all 135 students.[10]

Twelve to fourteen months later the dental examinations were repeated by

*_Procedure._ 0.5 c.c. of the saliva was added to 0.5 c.c. of a sucrose-indicator solution in a precipitation tube, bringing the concentration of sucrose to approximately 3.9 per cent. The sucrose-indicator solution was made up by adding to 1000 c.c. of distilled water, 80 Gm. of sucrose, 10 c.c. bromcresol purple, and 10 c.c. bromcresol green (both were 0.4 per cent indicator solutions made up with 95 per cent ethyl alcohol); the pH of the solution was adjusted to 6.6 with 4 c.c. N/20 NaOH. This made sufficient sucrose available for degradation, yet did not produce a great enough osmotic pressure to inhibit the metabolism of these microorganisms. Then the saliva-sucrose-indicator mixture (hereafter referred to as the "saliva-sucrose" mixture) was incubated at 37°C. for four hours and, after gentle agitation of the tube contents, the pH was determined by comparison with standard buffer-indicator solutions (pH values 6.4 and 6.0 were estimated by interpolation). These standard phosphate and phthalate-Hcl buffer solutions were made up at 0.4 pH intervals, ranging from pH 6.6 to pH 4.2. One cubic centimeter of each of these buffers was placed into small precipitation tubes to which 0.1 c.c. of bromcresol purple and 0.1 c.c. of bromcresol green were added (both 0.04 per cent indicator solutions made up with 95 per cent ethyl alcohol; then the standard buffer solutions ranged in color from deep blue to a bright yellow). Sharp color changes in these standard solutions at the 0.4 pH intervals resulted.

the original examiner. At no time was he aware of the laboratory test results performed at the time of the initial examinations.

RESULTS

The results of each individual's laboratory tests, and his dental caries experience during the subsequent year, are shown in Table I and suggest the following:

(a) The lower the pH of the "saliva-sucrose" mixture, the more dental caries occurred during the ensuing year; (b) the more total titratable acid produced, the greater was the dental caries increment; (c) the higher the L.a. index, the greater was the increase in dental caries.

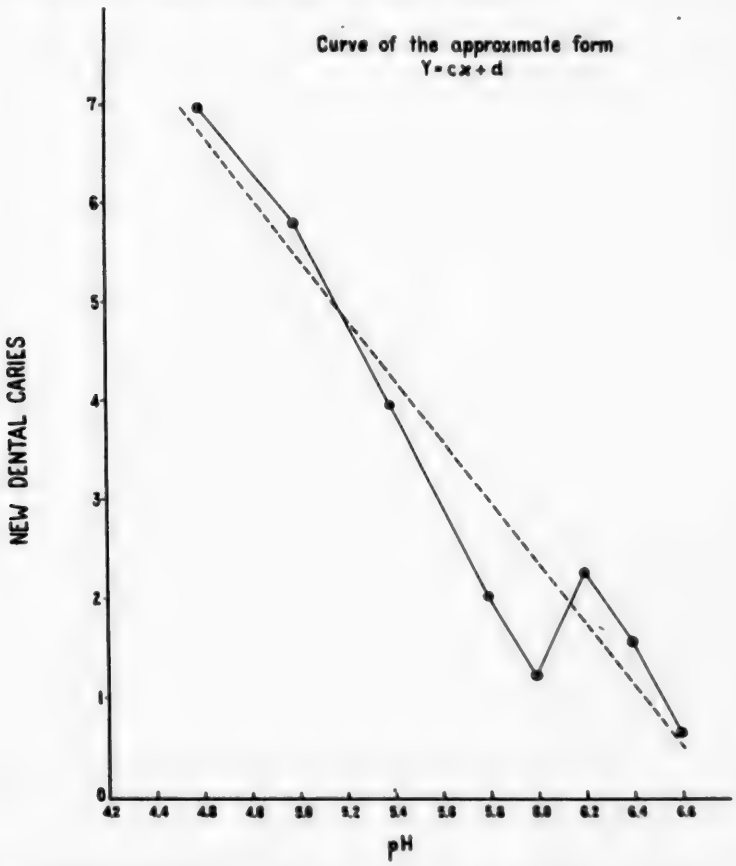

Fig. 1.—Relationship between new dental caries and pH of incubated saliva-sucrose mixtures.

The frequency with which new dental caries occurred at each of the pH values between 6.6 and 4.6 is shown in Table II.

Of the 6 individuals whose "saliva-sucrose" samples remained at a pH of 6.6, 3 had no newly attacked areas, 2 developed 1 new lesion, and 1 incurred 3,

TABLE I

INDIVIDUAL RELATIONSHIPS BETWEEN LABORATORY FINDINGS AND THE INCREASES IN DENTAL CARIES ONE YEAR AFTER TESTING

NO. OF INDIV.	TOTAL ACID*	pH†	L.a. INDEX‡	NEW DENTAL CARIES (SURFACES)	NO. OF INDIV.	TOTAL ACID*	pH†	L.a. INDEX‡	NEW DENTAL CARIES (SURFACES)
1	0.5	5.4	130,000	5	69		5.0	32,500	3
2	0.3	6.2	0	1	70		6.2	0	1
3	0.3	6.6	3,000	0	71	0.4	5.4	16,000	1
4	1.0	5.0	7,000	4	72	0.2	6.0	37,500	2
5	0.5	5.8	750,000	2	73	0.8	5.0	67,500	4
6	0.5	5.8	10,000	2	74	0.2	6.2	0	6
7	0.5	6.0	0	2	75	0.5	5.0	15,000	4
8	0.5	5.8	5,000	3	76	0.6	5.4	12,000	2
9	0.4	6.2	40,000	1	77	0.6	5.0	0	8
10	1.5	5.4	281,000	4	78	0.4	5.8	0	2
11	0.5	5.8	0	2	79	0.0	5.8	0	4
12	0.5	5.8	8,000	2	80	0.2	5.8	175,000	1
13	0.3	5.4	125,000	1	81	0.7	5.8	150,000	2
14	0.4	5.8	15,000	3	82	1.4	5.4	75,000	13
15	0.3	5.4	35,000	1	83		5.8	1,200	1
16	0.4	5.8	11,000	2	84		5.8	500	1
17	0.3	6.6	0	0	85	0.7	6.2	3,500	2
18	0.8	5.8	200,000	1	86	0.3	6.0	0	0
19	0.8	5.4	0	4	87	0.9	5.8	26,000	1
20	0.4	6.0	7,000	1	88	0.7	6.4	200	3
21	0.4	6.6	0	2	89	0.4	6.2	5,000	4
22	0.5	5.4	0	3	90	0.6	5.4	2,000	1
23	0.2	5.8	800	1	91	0.5	6.2	0	1
24	0.3	6.4	38,000	2	92	0.5	5.8	26,200	3
25	0.1	6.4	0	0	93	0.5	6.6	9,300	1
26	1.0	4.6	3,500	4	94	0.5	5.8	600	2
27	0.0	6.6	6,000	0	95	0.4	6.2	3,700	2
28	0.7	5.4	312,000	4	96	0.6	4.6	50,000	5
29	0.6	5.4	47,200	5	97	0.5	5.0	3,000	2
30	0.9	5.4	27,000	5	98	0.8	5.4	37,000	5
31	0.3	5.4	45,000	9	99	0.5	5.0	500,000	9
32	0.1	5.4	7,100	1	100	0.5	6.2	0	1
33	0.5	5.0	30,000	3	101	0.6	5.4	11,000	5
34	0.4	5.4	52,000	1	102	0.8	5.4	13,000	6
35	0.8	5.0	147,000	7	103	0.4	5.8	120,000	2
36	0.5	5.8	42,500	4	104	0.6	5.0	5,000	5
37	1.0	5.4	70,000	4	105	0.6	5.8	200	2
38	0.2	5.4	2,720	2	106	0.6	5.8	11,000	2
39	1.2	6.2	190,500	8	107	0.5	6.2	0	2
40	1.0	5.0	138,000	5	108	0.6	5.4	8,000	3
41	0.5	5.8	21,000	2	109	1.3	4.6	485,000	13
42	0.6	5.0	180,000	6	110	0.6	5.0	1,500	4
43	0.5	5.4	50,000	1	111	0.5	5.8	0	0
44	0.7	5.0	170,000	2	112	0.5	6.2	0	1
45	0.5	6.4	0	0	113	0.7	5.8	12,000	2
46			4,900	10	114	1.1	5.0	187,000	4
47	0.5	5.4	8,000	3	115	1.0	4.6	100	5
48	0.5	5.4	20,000	8	116	0.4	6.2	0	2
49	0.0	6.4	40,000	3	119	0.3	5.8	0	2
50	0.1	5.8	1,500	0	117	0.5	5.0	225,000	4
51	0.5	5.8	300	2	118	0.2	5.8	1,200	1
52	0.8	5.4	75,000	9	120	0.3	5.8	45,000	1
53	0.4	5.8	35,000	2	121	0.4	5.8	3,000	2
54	0.5	5.8	40,000	5	122	1.2	4.6	390,000	12
55	0.2	5.8	22,500	5	123	0.7	5.0	11,200	6
56	1.1	5.4	105,000	3	124	0.5	6.2	0	2
57	0.5	6.2	0	2	125	0.9	5.0	130,000	3

TABLE I—CONT'D

NO. OF INDIV.	TOTAL ACID*	pH†	L.a. INDEX‡	NEW DENTAL CARIES (SURFACES)	NO. OF INDIV.	TOTAL ACID*	pH†	L.a. INDEX‡	NEW DENTAL CARIES (SURFACES)
58	0.4	5.4	75,000	3	126	0.4	5.0	0	17
59	0.5	5.4	86,000	5	127	0.5	5.4	137,000	6
60	0.1	5.8	0	4	128	0.6	5.4	270,000	3
61	0.8	5.0	183,000	4	129	0.8	5.4	10,000	6
62	0.5	5.8	13,000	1	130	0.2	6.6	5,000	1
63		4.6	750,000	2	131	1.6	5.0	79,000	16
64	0.6	6.2	230	1	132	0.7	5.0	20,000	4
65	0.7	5.4	4,000	3	133	0.4	6.2	200	2
66	0.4	5.4	70,000	1	134	0.8	4.6	327,000	11
67	0.5	4.6	83,000	4	135	0.4	5.8	1,000	1
68	0.8	5.0	60,000	6					

*Total titratable acid of incubated "saliva-sucrose" samples; expressed in c.c. of 0.02 N NaOH.

†pH of "saliva-sucrose" samples after four hours' incubation.

‡Lactobacillus acidophilus indices.

TABLE II

FREQUENCY DISTRIBUTION OF NEW DENTAL CARIES AND THE pH OF THE INCUBATED "SALIVA-SUCROSE" MIXTURE

NEW DENTAL CARIES (SURFACES)	pH AT INITIAL EXAMINATION							
	6.6	6.4	6.2	6.0	5.8	5.4	5.0	4.6
0	3	2		1	2			
1	2		7	1	10	8		
2	1	1	7	2	17	3	1	1
3		2			4	6	3	
4			1		3	4	8	2
5					2	6	2	2
6			1			3	3	
7							1	
8			1			1	1	
9						2	1	
11								1
12								1
13						1		1
16							1	
17							1	
Σ	4	8	39	5	78	135	128	56
N	6	5	17	4	38	34	22	8
M	0.67	1.60	2.29	1.25	2.06	3.97	5.83	7.0

making a total (Σ) of 4 and a mean (M)* of 0.67 new carious areas. In contrast, of the 8 students whose "saliva-sucrose" samples dropped to a pH of 4.6, only 1 incurred 2 lesions while 2 developed 4, and 2 others each had 5 more. One student had 11 additional carious surfaces, while 2 others developed 12 and 13 new lesions, respectively. For this group a mean (M) increase of 7.0 new carious surfaces was evidenced. Fig. 1 shows the almost linear relationship between decreasing pH and increasing mean dental caries (surfaces).

The curve takes the general form $Y = CX + d$, in which Y is equivalent to M, C and d are constants, and X represents the pH values.

As far as titratable acid is concerned, the frequency of new dental caries (surfaces) is recorded for each 0.1 c.c. interval, in Table III.

*Mean (M) = average increase in dental caries per individual.

One individual who originally had no titratable acid incurred no new lesions at the end of the year, 2 others developed 3 and 4 lesions, respectively; i.e., 7 newly attacked areas were developed by 3 students to give a mean (M) increase of 2.33 carious lesions. On the other hand, 8 students demonstrating 1.1 c.c. or more of total titratable acid (Table III, last column) developed 3,4,4,8,12,13,13, and 16 new carious lesions, respectively, a mean increase of 9.13 areas. Fig. 2 illustrates the relationship between each 0.1 c.c. rise in total titratable acid and mean new dental caries. This approximate curve takes the parabolic form $Y = CZ^2$, in which mean dental caries increment seems to increase directly with the square of the total titratable acid.

TABLE III

FREQUENCY DISTRIBUTION OF NEW DENTAL CARIES AND THE TOTAL TITRATABLE ACID OF THE INCUBATED SALIVA-SUCROSE MIXTURES

NEW DENTAL CARIES (SURFACES)	TOTAL TITRATABLE ACID AT INITIAL EXAMINATION											
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1
0	1	2		3		2						
1		1	4	4	5	6	2		1	1		
2			2	2	9	12	3	4				
3	1				2	5	2	2		1		1
4	1	1			1	4	1	2	3		3	2
5			1			3	4		1	1	2	
6			1			1	1	1	3			
7									1			
8						1	1					1
9			1	1		1			1			
11										1		
12												1
13												2
16												1
17					1							
Σ	7	5	28	17	50	99	52	28	52	20	22	73
N	3	4	9	10	18	35	14	9	10	4	5	8
M	2.33	1.25	3.11	1.70	2.78	2.82	3.72	3.11	5.2	5.0	4.4	9.13

In Table IV the frequency with which new dental caries occurred is shown for each of the L.a. indices (also expressed as logarithms of the indices).

Of the 27 individuals whose L.a. indices fell in the lowest range, from 1* to 100, 5 had no new lesions at all. Five other students developed only 1 and 9 incurred 2, 1 had 3, and 3 had 4 newly attacked surfaces. However, there were a few with 5, 6, 8, or even 17 more areas involved. The total number of new dental caries (Σ) was 74, resulting in a mean (M) increase of 2.74. In contrast, the 26 individuals whose L.a. indices fell into the highest range, from 100,000 to 1,000,000, developed a total (Σ) of 124 lesions, resulting in a mean (M) of 4.77 new dental caries (surfaces), which is almost twice that of the lowest L.a. index group.

Fig. 3 demonstrates the approximately parabolic relationship ($Y = CW^2$) between mean new dental caries and the logarithms of the L.a. indices. The

*L.a indices of 1 were used when the actual value was 0, in order to make logarithmic computations possible. The log of 0 is infinity, while the log of 1 is 0, which can be plotted on a graph. There is of course no practical difference between an index of 1 and 0.

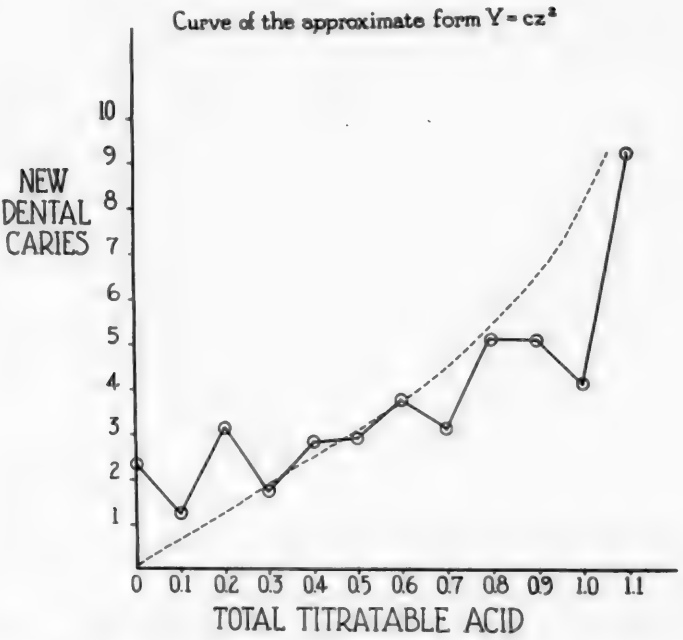

Fig. 2.—Relationship between new dental caries and total titratable acid of incubated saliva-sucrose mixtures.

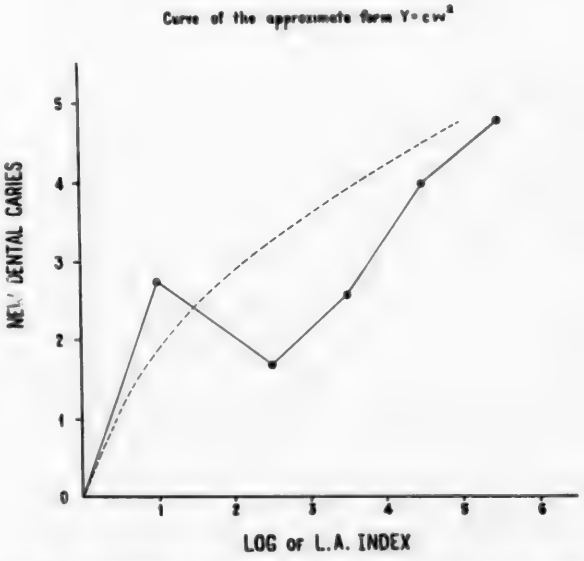

Fig. 3.—Relationship between new dental caries and L.a. indices.

TABLE IV

FREQUENCY DISTRIBUTION OF NEW DENTAL CARIES AND L.a. INDUCES OR THEIR LOGARITHMS

NEW DENTAL CARIES (SURFACES)	L.a. INDICES AT TIME OF INITIAL EXAMINATION				
	1-100	100-1,000	1,000-10,000	10,000-100,000	100,000-1,000,000
	LOGARITHMS OF L.a. INDICES				
	0-2	2-3	3-4	4-5	5-6
0	5		3		
1	5	4	6	10	3
2	9	4	7	8	5
3	1	1	5	5	3
4	3		4	6	5
5	1		1	8	2
6	1		1	3	2
7					1
8	1			1	1
9				2	1
10			1		
11					1
12					1
13				1	1
16				1	
17	1				
Σ	74	15	72	178	124
N	27	9	28	45	26
M	2.74	1.67	2.57	3.97	4.77

mean values for each range of the logarithms of the L.a. indices were plotted at the midpoint of the range, e.g., the midpoint in the lowest range is halfway between the logarithm 0 and the logarithm 2, or logarithm 1; therefore, the mean increase in dental caries (M) of 2.74 is plotted opposite the log, 1.

A statistical analysis of the correlation of each of the three tests to new dental caries and to each other was then made. The following symbols were assigned:

Y = actual number of new carious lesions; Y_p = predicted number of new carious lesions

X = pH of the "saliva-sucrose" mixtures

Z = total titratable acid (in c.c. of 0.02 N NaOH)

W = L.a. index

Because of the relationships between Y and each of the other quantities as shown in the approximate curves in Figs. 1, 2, and 3, $X_1 = X$, $X_2 = Z^2$, and $X_3 = (\log W)^2$.

The best individual linear relationships between Y and X_1, X_2, X_3, respectively, may be expressed in the form of the following regression equations:

(1) $Y_{p1} = 22.3 - 3.4\ X_1$ (predicted new carious lesions on the basis of the pH determination)

(2) $Y_{p2} = 1.8 + 4.1\ X_2$ (predicted new carious lesions on the basis of total titratable acid determination)

(3) $Y_{p3} = 2.0 + 0.10\ X_3$ (predicted new carious lesions on the basis of the L.a. index determination)

Y_{p1}, Y_{p2}, and Y_{p3} are the Y_p's or new dental caries predicted on the basis of X_1, X_2, or X_3. Furthermore, the best linear relationship between Y_p and X_1, X_2, and X_3 taken collectively is:

$Y_p = 15.7 - 2.4\ X_1 + 3.0\ X_2 - 0.01\ X_3$, in which 15.7, 2.4, 3.0, and 0.01 are the partial regression coefficients b_0, b_1, b_2, and b_3,* respectively. The 95 per cent confidence limits for the b's are: $b_0 \pm 5.70$, $b_1 \pm 0.93$, $b_2 \pm 1.05$, $b_3 \pm 0.047$. The random variable $\frac{b_3 - B_3}{S_{b_3}}$ is approximately normally distributed with mean 0 and standard deviation = 1 (S_{b_3} = standard

*b_1, b_2, and b_3 are the partial regression coefficients, estimated from the data, of which the true partial regression coefficients are B_1, B_2 and B_3, respectively.

error of b_3). b_3 is 0.00964 (0.01) and $S_{b_3} = 0.0238$. Since $\frac{b_3}{S_{b_3}} = 0.41$, b_3 is not significantly different from 0 and should not be included in the equation. Consequently, X_3, and therefore, the L.a. indices, should not be used when all three tests are jointly employed in the estimation of Y_p. However, b_1 and b_2, and B_1 and B_2 are significantly different from 0 and therefore X_1 and X_2 are justifiably used.

The corrected regression equation, excluding X_3, is $Y_p = 15.1 - 2.3\ X_1 + 2.9\ X_2$, where 15.1, 2.3, and 2.9 are regression coefficients b_0, b_1, and b_2, respectively. The 95 per cent confidence limits for these b's are: $b_0 \pm 5.02$, $b_1 \pm 0.86$, $b_2 \pm 1.02$.

Correlations, or the degree of linear relationship among the involved quantities are:

$r12$ (correlation coefficient between X_1 and X_2) $= -0.44$
$r13$ (correlation coefficient between X_1 and X_3) $= -0.47$
$r23$ (correlation coefficient between X_2 and X_3) $= 0.38$
$r1Y$ (correlation coefficient between X_1 and Y) $= -0.56$
$r2Y$ (correlation coefficient between X_2 and Y) $= 0.57$
$r3Y$ (correlation coefficient between X_3 and Y) $= 0.31$

All of these correlations are so large that they cannot be ascribed to chance, although they are not large enough for one to expect a highly accurate prediction of Y (Y_p) on the basis of X_1, X_2, X_3. Furthermore, the nonsignificance of the partial regression coefficient b_3 connotes the nonsignificance of the partial correlation $rYX_3 \cdot X_1X_2$, while the significances of the partial regression coefficients b_1 and b_2 result in the significant partial correlations $rYX_1 \cdot X_2X_3$ and $rYX_2 \cdot X_1X_3$, respectively. In other words, the fact that the partial correlation $rYX_1 \cdot X_2X_3$ is significant means that in four-dimensional space, if X_2 and X_3 are held constant, a fixed plane results, in which linear relationships exist between Y and X_1. The significant partial correlation $rYX_2 \cdot X_1X_3$ is similarly interpreted. However, the nonsignificance of $rYX_3 \cdot X_1X_2$ means that when X_1 and X_2 are held constant, there are no linear relationships between Y and X_3 in the resulting plane. In restatement, any linear relationships between Y and X_1, X_2, and X_3 (taken collectively) depend on X_1 and X_2, but not X_3. This implies that X_3 and, therefore, the L.a. index, has its relationship with Y, or new dental caries, only via X_1 and X_2, or the pH and total titratable acid, respectively, a finding which may be explained on other than statistical grounds.

Errors of prediction:

A. Standard errors of predictions (Y_p's) for each of the three tests used singly:
(1) $S_{Yp1} = 2.57\ [1 + (9.54)(10^{-1}) + (3.01)(10^{-2})(X_1)^2 - (3.37)(10^{-1})(X_1)]^{1/2}$
(2) $S_{Yp2} = 2.55\ [1 + (1.43)(10^{-2}) + (4.23)(10^{-2})(X_2)^2 - (3.32)(10^{-2})(X_2)]^{1/2}$
(3) $S_{Yp3} = 2.95\ [1 + (2.75)(10^{-2}) + (8.08)(10^{-3})(X_3)^2 - (2.53)(10^{-3})(X_3)]^{1/2}$

B. Standard error of prediction (Y_p) if X_1 and X_2 are used jointly:
$S_{Yp} = 2.28\ [1 + 1.27 + (3.72)(10^{-2})(X_1)^2 + (5.23)(10^{-2})(X_2)^2 - (4.32)(10^{-1})(X_1) - (2.57)(10^{-1})(X_2) + (3.85)(10^{-2})(X_1)(X_2)]^{1/2}$

Calculation of Y_p and various confidence intervals in which the true Y would lie:

(1) Y_p is calculated from the appropriate regression equation.
(2) S_{Yp} is calculated from the corresponding equation for standard error of prediction.
(3) The confidence limits are established:
(a) 95 per cent confidence intervals have the form ($Y_p \pm 1.96\ S_{Yp}$)
(b) 68 per cent confidence intervals have the form ($Y_p \pm 1.00\ S_{Yp}$)
(c) 50 per cent confidence intervals have the form ($Y_p \pm 0.67\ S_{Yp}$)

Example: If the pH of an individual's "saliva-sucrose" sample were 5.0, then,
$X = 5.0$, $X_1 = X = 5.0$
$Y_{p1} = 22.3 - (3.4)(X_1) = 22.3 - (3.4)(5.0) = 5.3$ (predicted new lesions)
$S_{Yp1} = 2.57\ [1 + (9.54)(10^{-1}) + (3.01)(10^{-2})(X_1)^2 - (3.37)(10^{-1})(X_1)]^{1/2}$
$= 2.57\ [1 + (9.54)(10^{-1}) + (3.01)(10^{-2})(25) - (3.37)(10^{-1})(5)]^{1/2}$
$= 2.58$
95 per cent confidence intervals $= Y_{p1} \pm (1.96)(S_{Yp1}) = 5.3 \pm (1.96)(2.58) = 0.24$ to 10.36 or 0 to 10 new carious surfaces

68 per cent confidence intervals $= Y_{p1} \pm (1.00)(S_{Yp1}) = 5.3 \pm 2.58 = 2.72$ to 7.88 or 3 to 8 new carious surfaces

50 per cent confidence intervals $= Y_{p1} \pm (0.67)(S_{Yp1}) = 5.3 \pm (0.67)(2.58) = 3.64$ to 6.96 or 4 to 7 new carious surfaces

Similarly, the predicted increases in dental caries (Y_{p1}) and the confidence intervals for each 0.4 unit fall in pH was calculated as shown in Table V.

An almost linear relationship exists between Y_{p1} and X_1, with a pH of 6.6 giving a predicted increase of 0 (−0.1, theoretically) carious lesions, while a pH of 4.2 forecasts 8. The predicted increments (Y_{p1}'s) will only rarely be the true increases (Y_1's); these will lie between certain limits determined by the confidence level chosen. As shown in the example for 95 per cent confidence, with a pH of 5.0, the interval in which Y will be found is about 10 units (0 to 10 new lesions). However, for 68 per cent accuracy in predicting the increase in dental caries from the same pH value, a shortening of the interval to 5 occurs. In the example just given, then, if a patient's "saliva-sucrose" mixture demonstrates a pH of 5.0, the chances are better than 2 out of 3 that he will develop between 3 and 8 new lesions in the coming year. At the 50 per cent confidence level the interval is reduced to 3; i.e., there is an even chance that the individual will incur between 4 and 7 new dental caries.

The predicted increases in dental caries and confidence intervals based on the total titratable acid (Z) of the incubated "saliva-sucrose" mixtures are shown in Table VI. Although the values for the predicted new dental caries (surfaces) (Y_{p2}) rise with those for the total titratable acid (X_2), the relationship is parabolic, as was determined by evaluation of the original data. A total titratable acid of 0 predicts approximately 2 new lesions during the ensuing year; 0.9 c.c. would predict an increase (Y_{p2}) of 5 more carious areas; at the 95 per cent confidence level, the interval in which the true increase (Y_2) may fall ranges from 0 to 10; at the 68 per cent confidence level this interval is halved, to 3 to 8 new lesions; while at the 50 per cent level, there is an even chance that between 3 and 7 more areas may develop.

L.a. indices (W) with their corresponding dental caries increments (Y_{p3}'s) and confidence intervals are shown in Table VII. An L.a. index of 0 (assigned a value of 1 for reasons given previously) results in a predicted increase of 2 new lesions. When an individual's L.a. index is 500,000 the predicted increase in dental caries (Y_{p3}) would be 5.25, and the true increase (Y_3) could be anywhere between 0 and 11 new lesions at 95 per cent confidence. At 68 per cent and 50 per cent confidence levels, it is seen that the true increase in dental caries lies between 2 and 8, and 3 and 7 new lesions, respectively. It is noted that the intervals at all 3 confidence levels are 1 to 2 units larger, and correspondingly less precise, than the intervals in Tables V and VI.

DISCUSSION

The original data indicate definite correlations between each of the three prognostic tests and the actual increment in dental caries and among the individual tests. The significance and description of these relationships were elucidated by statistical methods.

It is highly probable that the use of averages for each of the three tests obtained from several saliva samples from each individual could have resulted in closer correlations between each of the tests and dental caries increment, and among the tests themselves. This probability is now being investigated. However, it is important to note that, in this preliminary study, significant, though not highly specific, relationships were obtained on the basis of single tests. The findings in this investigation demonstrate the definite relationship of dental caries increment with the acidogenic potential of saliva as measured either by the pH or the total titratable acid of incubated "saliva-sucrose" mixtures.

Table V

Probable Dental Caries Increment During One Year; Predicted From the pH of Incubated "*Saliva-Sucrose*" Samples

pH of Sample	Predicted Dental Caries (Surfaces)	Confidence Limits					
		95%		68%		50%	
		Theoretical Range	Practical Range	Theoretical Range	Practical Range	Theoretical Range	Practical Range
6.6	–0.1	–5.24 to 5.04	0 to 5	–2.72 to 2.52	0 to 3	–1.86 to 1.66	0 to 2
6.4	0.5	–4.6 to 5.6	0 to 6	–2.1 to 3.1	0 to 3	–1.24 to 2.24	0 to 2
6.2	1.2	–3.9 to 6.3	0 to 6	–1.4 to 3.8	0 to 4	–0.54 to 2.94	0 to 3
6.0	1.9	–3.16 to 6.96	0 to 7	–0.68 to 4.48	0 to 4	0.23 to 3.56	0 to 4
5.8	2.6	–2.46 to 7.66	0 to 8	0.02 to 5.18	0 to 5	0.94 to 4.26	1 to 4
5.4	3.9	–1.16 to 8.96	0 to 9	1.32 to 6.48	1 to 6	2.24 to 5.56	2 to 6
5.0	5.3	0.24 to 10.36	0 to 10	2.72 to 7.88	3 to 8	3.64 to 6.96	4 to 7
4.6	6.7	0.56 to 11.84	1 to 12	4.08 to 9.32	4 to 9	4.94 to 8.46	5 to 8
4.2	8.0	2.81 to 13.19	3 to 13	5.35 to 10.65	5 to 11	5.88 to 10.12	6 to 10

Table VI

Probable Dental Caries Increment During One Year; Predicted From Values for Total Titratable Acid of Incubated "*Saliva-Sucrose*" Mixtures

Total Titratable Acid	Predicted Dental Caries (Surfaces)	Confidence Limits					
		95%		68%		50%	
		Theoretical Range	Practical Range	Theoretical Range	Practical Range	Theoretical Range	Practical Range
0	1.8	–3.23 to 6.83	0 to 7	–0.76 to 4.36	0 to 4	0.08 to 3.52	0 to 4
0.1	1.84	–3.19 to 6.87	0 to 7	–0.72 to 4.46	0 to 4	0.12 to 3.56	0 to 4
0.2	1.96	–3.07 to 6.99	0 to 7	–0.60 to 4.56	0 to 5	0.24 to 3.68	0 to 4
0.3	2.17	–2.86 to 7.20	0 to 7	–0.39 to 4.73	0 to 5	0.45 to 3.89	0 to 4
0.4	2.46	–2.59 to 7.49	0 to 7	–0.10 to 5.02	0 to 5	0.74 to 4.18	1 to 4
0.5	2.83	–2.20 to 7.86	0 to 8	0.27 to 5.39	0 to 5	1.11 to 4.55	1 to 5
0.6	3.28	–1.75 to 8.31	0 to 8	0.72 to 5.84	1 to 6	1.56 to 5.00	2 to 5
0.7	3.82	–1.21 to 8.85	0 to 9	1.26 to 6.38	1 to 6	2.10 to 5.54	2 to 6
0.8	4.42	–0.6 to 9.45	0 to 9	1.86 to 6.98	2 to 7	2.70 to 6.14	3 to 6
0.9	5.12	0.08 to 10.16	0 to 10	2.55 to 7.69	3 to 8	3.39 to 6.85	3 to 7
1.0	5.9	0.84 to 10.96	1 to 11	3.32 to 8.48	3 to 8	4.17 to 7.63	4 to 8
1.1	6.76	1.69 to 11.83	2 to 12	4.17 to 9.35	4 to 9	5.02 to 8.50	5 to 9

TABLE VII

PROBABLE DENTAL CARIES INCREMENT DURING ONE YEAR; PREDICTED FROM L.A. INDICES

L.a. INDEX	PREDICTED DENTAL CARIES (SURFACES)	CONFIDENCE LIMITS					
		95%		68%		50%	
		THEORETICAL RANGE	PRACTICAL RANGE	THEORETICAL RANGE	PRACTICAL RANGE	THEORETICAL RANGE	PRACTICAL RANGE
1	2.00	−3.86 to 7.86	0 to 8	−0.99 to 4.99	0 to 5	0.00 to 4.00	0 to 4
100	2.40	−3.46 to 8.26	0 to 8	−0.59 to 5.39	0 to 5	0.40 to 4.40	0 to 4
1,000	2.90	−2.92 to 8.72	0 to 9	−0.07 to 5.87	0 to 6	0.91 to 4.89	1 to 5
10,000	3.60	−2.17 to 9.37	0 to 9	0.65 to 6.55	1 to 7	1.62 to 5.58	2 to 6
50,000	4.21	−1.61 to 10.03	0 to 10	1.24 to 7.18	1 to 7	2.02 to 6.20	2 to 6
100,000	4.50	−1.32 to 10.32	0 to 10	1.53 to 7.47	2 to 7	2.51 to 6.47	3 to 6
500,000	5.25	−0.63 to 11.13	0 to 11	2.25 to 8.25	2 to 8	3.24 to 7.26	3 to 7
1,000,000	5.60	−0.30 to 11.50	0 to 12	2.59 to 8.61	3 to 9	3.58 to 7.62	4 to 8

Furthermore, the conclusion of many investigators that the L.a. index is closely associated with dental caries activity (as measured by dental caries increment) is borne out. On the other hand, it is noteworthy that the statistical consultant, with only minimal comprehension of the clinical aspects of the study, found that when all three tests were used, the deletion of the L.a. index made the prediction of the number of new dental caries (surfaces) no less precise. Moreover, he found that, statistically, the subsequent dental caries increment could be consistently correlated to the L.a. index only through the pH and total titratable acid of the "saliva-sucrose" mixtures. (It must be stated that in the past the L.a. index was used primarily as an "index" of present caries activity and not of future dental caries increment.)

Besides establishing the variation in the acid-producing abilities of strains of lactobacilli, several investigators have shown that streptococci, micrococci, yeasts, etc., may also contribute to the total acidogenic potential of microorganisms in saliva.[7, 9, 11, 13, 14, 16, 18-21, 23, 24, 25] Since the prevailing view on the pathogenesis of dental caries is that it seems to be dependent on acid production, it would appear that the determination of the total acidogenic potential of micrograms in saliva may be of greater practical interest than the estimation of the numbers of only one acidogen.

Since it was possible by statistical technics to predict the dental caries increment within certain limits, the increase in carious lesions was calculated for three levels of confidence. The 95 per cent level is too high for efficient use; on the other hand, that of 50 per cent is not sufficiently reliable. The chances of the 68 per cent confidence level being correct are better than two out of three, and at this level (as on the other two) it may be seen that the intervals of dental caries increments are at least one unit smaller for total titratable acid and pH than for the L.a. index. If further investigation bears out the findings in this study, it would seem to indicate that the acidogenic tests, in addition to present tests for dental caries activity, may be used in the prediction of new annual dental caries.

Both of these acidogenic tests appear to be of practical importance to the individual practitioner because of their somewhat greater prognostic accuracy and simplicity of performance when compared to previous tests. Furthermore, since the colorimetric pH determination is simpler, much more adaptable to the dental office, and is less expensive than the determination of total titratable acid, it offers to the general dental practitioner a possible new tool for the determination of dental caries activity on the basis of probable dental caries increment, and may thereby indicate proper therapy.

In the field of clinical research, investigators might find this test useful in separating caries-active from caries-inactive individuals or in testing the effectiveness of a substance for its ability to reduce salivary acid production in vitro.

Although future investigations utilizing several salivary analyses may yield more precise predictions, the development of this new colorimetric pH determination permits the tentative classification shown in Table VIII of dental caries activity on the basis of the probable subsequent dental caries increment.

TABLE VIII

pH*	PREDICTED NEW DENTAL CARIES AFTER 1 YEAR (68 PER CENT CONFIDENCE LEVEL)	DEGREE OF ACTIVITY
6.6	0 (0-3)	Low
6.2	1 (0-4)	
5.8	3 (0-5)	Moderate
5.4	4 (1-6)	
5.0	5 (3-8)	High
4.6	7 (4-9)	
4.2	8 (5-11)	

*pH of "saliva-sucrose" mixture after four hours of incubation.

SUMMARY

Three different tests for dental caries activity were performed to determine their value in the prediction of subsequent annual dental caries increment. Individual saliva samples of 129 University of California students were all analyzed for (1) a new colorimetric pH determination, (2) total titratable acid, and (3) the *Lactobacillus acidophilus* index.

One year later the increment of dental caries for each individual was determined and plotted against the results of these tests. Employing statistical methods, it was found that the increase in dental caries could be predicted within definite limits on the basis of each of the tests and that they had significant correlations with each other. The colorimetric pH determination method, however, appeared to be the most accurate, simple, and effective procedure which may be used in routine office practice.

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A REPRODUCIBLE METHOD FOR CARIES EVALUATION

III. Test in a Therapeutic Experiment With an Ammoniated Dentifrice

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INTRODUCTION

IN A previous publication[1, 2] we stressed the poor reproducibility of clinical caries evaluation, leading to large observation errors in caries experiments. A roentgenographic method for the diagnosis of early caries lesions of the proximal surfaces of molars and premolars was described[1] and tested.[2] In this paper the method is further tested in an evaluation of the prophylactic value of an ammoniated dentifrice.

The observations of Stephan[3] and Kesel, O'Donnell, Kirch, and Wach,[4] concerning the effect of urea and ammonia on neutralization and production of acid in the plaque gave rise to much experimental work on the theoretical[5-8] as well as on the clinical side. The effect of urea in various concentrations, with or without ammonium salts, has already been studied in several clinical experiments (Stephan and Miller,[9] Goodfriend,[10] Henschel and Lieber[11]).

In 1947 Kesel, O'Donnell, Kirch, and Wach,[12] largely from the results of in vitro experiments, advocated the use of a powder and mouth rinse containing 5 per cent dibasic ammonium phosphate and 3 per cent urea. In a first clinical trial a favorable effect on lactobacillus counts was reported.[13] A carefully supervised experiment, on a large group of children, showed practically no beneficial effect of these prophylactic agents on the caries increase.[14]

Davies and King[15] arrived at the same results in a group of young people of the age 12 to 31; in this experiment brushing of the teeth was not highly supervised and therefore more in agreement with the common use of dentifrices. However in this study the caries increment in both groups, expressed as D.F. tooth surfaces, was 6.43 during an experimental period of 8 months, which is 9.64 per annum. For this age group this is a very large increase which seems to indicate that these patients must have been highly caries susceptible, as in the hands of these authors a shift of standards seems improbable.

Bibby and Nevin[16] did not find a reduction of lactobacillus counts in a group of 25 women students after the use of an ammoniated dentifrice over a period of 7 months.

Our experiment, started in 1949, can only confirm these results. Publication of our results seemed worth while, however, as another method of caries evaluation was used, which seems very reliable and reproducible. Furthermore a carefully selected group of patients between 10 and 14 years was studied. A tooth paste was used instead of tooth powder or mouth rinse.

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MATERIALS AND METHODS

Caries Evaluation.—We strictly adhered to the method described in a previous paper.[1] Duplicate series of bite wing roentgenograms were taken from the left and right premolar and molar region, with an apparatus which fixes the film in the mouth in a standard position, so as to obtain identical roentgenograms at different times. Caries was evaluated by two of us separately viewing the roentgenograms against illuminated milk glass through a fixed lens. The separate results were checked and combined. It was shown that the reproducibility of the roentgenograms is about 96 per cent, while caries could be estimated with a standard error of the mean of 0.3 per cent.

Lactobacilli in saliva were estimated by viable count on tomato agar of pH 5 using the rolling tube technique developed by Julius[17] in this laboratory. Saliva was obtained by chewing paraffin before breakfast. Five classes were recognized: Class I with 0 to 500 viable lactobacilli per ml., Class II 500 to 5,000, Class III 5,000 to 50,000, Class IV 50,000 to 500,000, and Class V more than 500,000.

Patients.—The experiment was started with 234 boys of 10 to 14 years. A duplicate set of roentgenograms was taken of each boy and the number of lactobacilli in saliva was estimated twice. The boys in each school were then paired, each pair comprising two boys of about the same age, the same number and site of cavities, the same number of deciduous and permanent teeth, and in the same class of lactobacilli. One boy in each pair was assigned to the red group, the other to the blue group. The boys were then instructed in brushing their teeth (twice daily) without rinsing after the procedure; an instruction card was fastened above each washstand. The boys were supplied with a red or blue toothbrush and a supply of tooth paste in red or blue tubes. The brushing was supervised by the teaching personnel of each school. Calculated from the amount of tooth paste supplied, a mean of 1 Gm. of tooth paste was used daily, in accordance with instructions. Further details on these procedures can be found in a previous paper.[2] The experiment was completed with 83 pairs (166 boys), mean age 12.8 ± 0.1

Dentifrice.—The tooth paste was prepared by Dobbelman N.V. and contained:

	Red	Blue
Calcium carbonate (precipitated)	32	30
Glycerol	11.3	12
Carboxymethylcellulose	2.7	1.9
Wetting agent	2.3	2.3
Sodium citrate	13	11
Flavoring agents	1.3	1.3
Diammonium phosphate	-	5
Urea	-	3
Water	37.5	34

Which paste contained the prophylactic agent was only disclosed at the end of the experiment.

RESULTS

Caries was evaluated as described before. Each roentgenogram was studied by two of us (O.B.D. and J.v.A.) and the results recorded on separate charts.

The charts were then compared, differences checked by both dentists jointly, and the combined results charted again. These final charts were marked I^{AC} and I^{BC} for the duplicate series of roentgenograms taken at the start of the experimental period and II^{AC} and II^{BC} for those taken 18 months later. Caries incidence for each type of a surface and standard errors were calculated from these charts as described before.[1] All results refer to the 83 pairs of boys who completed the experiment.

Caries Incidence.—Table I gives the number of surfaces, carious surfaces, and the standard error of the mean for each type of surface in the right maxillary half; the other three quadrants gave analogous results, which are not presented in detail, but included in the subtotal T_8S for the left maxillary half, T_{16} max. for the maxillary teeth, T_{16} mand. for the mandibular teeth, and T_{32} for all surfaces.

TABLE I. CARIES INCIDENCE AND STANDARD DEVIATIONS

	RIGHT MAXILLA									T_8S LEFT MAX.	T_{16} MAX.	T_{16} MAND.	T_{32} MAX. + MAND.
	M_2		M_1		P_2		P_1						
	D	M	D	M	D	M	D	M	T_8D				
RED, CARIES I													
Surfaces	13	42	73	67	53	63	69	41	421	432	853	789	1642
Caries	0	4	18	53	30	21	22	3	153	170	324	318	642
Per cent caries	4	11	25	79	57	34	33	8	37	39	38	40	39
Standard error of the mean (per cent)	5.4	1.7	2.3	2.1	3.2	3.6	1.7	2.9	1.0	0.9	0.7	0.7	0.5
RED, CARIES II													
Surfaces	13	42	73	67	53	63	69	41	421	432	853	789	1642
Caries	1	21	39	61	42	42	44	11	263	258	521	510	1032
Per cent caries	11	50	53	91	80	67	64	28	63	60	61	65	63
Standard error of the mean (per cent)	5.4	3.3	3.3	2.5	3.0	3.0	2.5	2.9	1.1	1.1	0.8	0.8	0.6
Increase (per cent)	8	39	29	12	24	33	31	19	26	20	23	24	24
BLUE, CARIES I													
Surfaces	12	42	73	72	55	63	70	47	434	455	889	787	1676
Caries	0	7	23	48	27	26	27	3	163	183	346	320	667
Per cent caries	0	18	32	67	49	41	39	6	37	40	39	41	40
Standard error of the mean (per cent)	0	3.8	3.1	2.2	3.1	2.7	2.9	2.6	1.1	1.1	0.8	0.8	0.5
BLUE, CARIES II													
Surfaces	12	42	73	72	55	63	70	47	434	455	889	787	1676
Caries	1	19	49	62	45	39	42	10	267	270	538	525	1063
Per cent caries	8	45	67	87	82	62	61	21	61	59	61	67	63
Standard error of the mean (per cent)	0	3.3	3.3	1.9	3.6	2.7	3.6	3.6	1.2	1.1	0.8	0.8	0.6
Increase (per cent)	8	27	35	19	33	21	22	14	24	19	22	26	24

Key: Caries I = caries incidence at start of experimental period
Caries II = caries incidence at end of experimental period

M_1 = first molar
P_2 = second premolar
m = mesial surface
d = distal surface

T = total, T_8S = total for the eight surfaces of the left (S) maxilla.
The caries numbers obtained by our method of calculation are not always whole numbers, but often end in halves. In the tables only whole numbers are given. Caries percentages are given without decimal fractions. This explains why totals do not always tally.

Caries incidence at the start (Caries I) and at the end (Caries II) of the experimental period is given for the red and blue groups.

Caries increase is given separately. The data of Table I are reproduced in graphic form in Fig. 1 (caries incidence) and Fig. 2 (caries increase). Table I and Figs. 1 and 2 show:

A. The caries incidence at the start of the experiment was equal in the red and blue groups, not only with regard to total numbers of cavities but also with regard to the distribution over various surfaces.

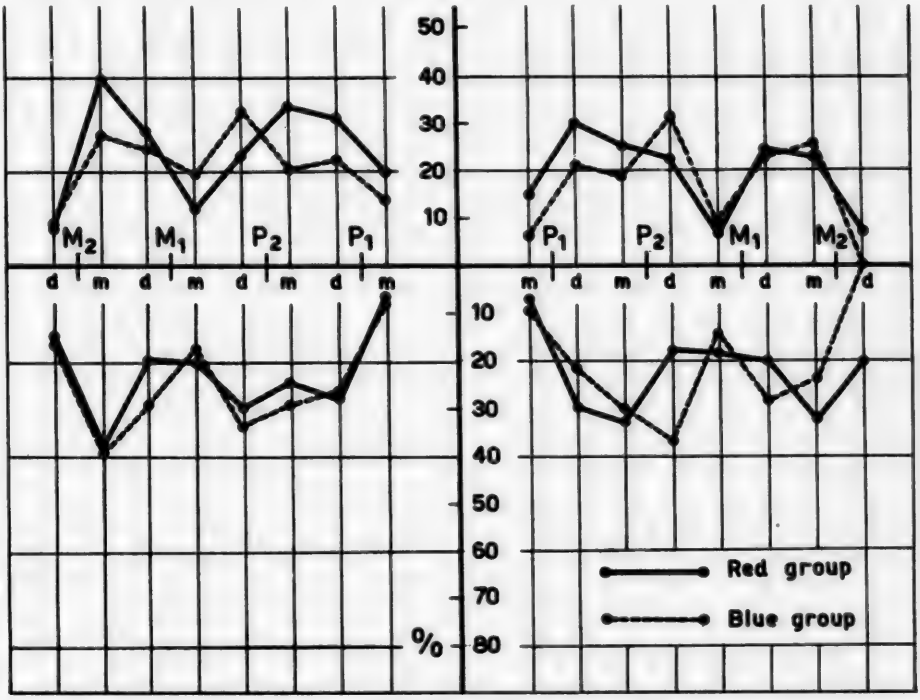

Fig. 1.—Per cent of proximal caries in each type of surface at the start (Caries I) and at the end (Caries II) of the experimental period (Table I).

B. A marked increase in caries incidence during the experimental period is observed in all surfaces. Within the experimental error no difference between the red and blue groups is observed (Figs. 1 and 2). These results are also given in summary form in Table IV.

New Caries in Sound Surfaces.—One of the best ways to express caries increase is to study the new cavities occurring during the experimental period in surfaces which were evaluated "sound" at the start. Caries incidence in sound surfaces was calculated from our data and the results are given in Table II and Fig. 3. Table II is arranged in the same way as Table I, giving full particulars of one maxillary half and subtotals for other quadrants. As the number of sound surfaces is much smaller than the total number of surfaces the results

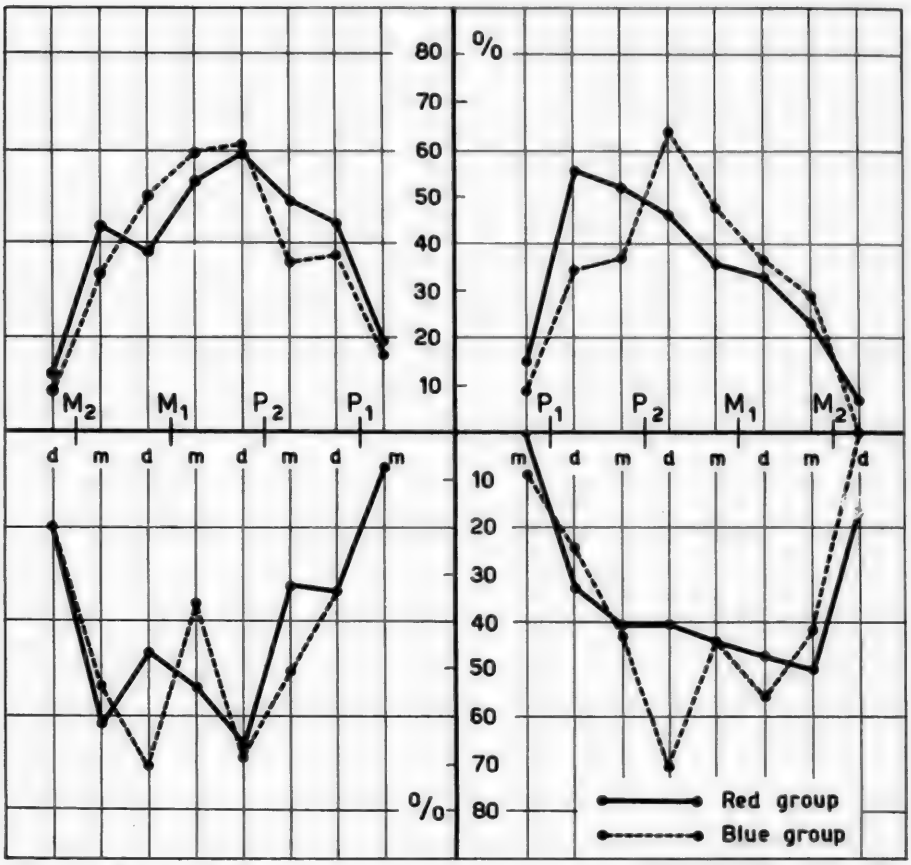

Fig. 2.—Per cent of new proximal caries in each type of surface (= caries increase =Caries II – Caries I). See Table I.

The value for Mid in the right maxillar half was erroneously put at 25 per cent; it should be at 35 per cent.

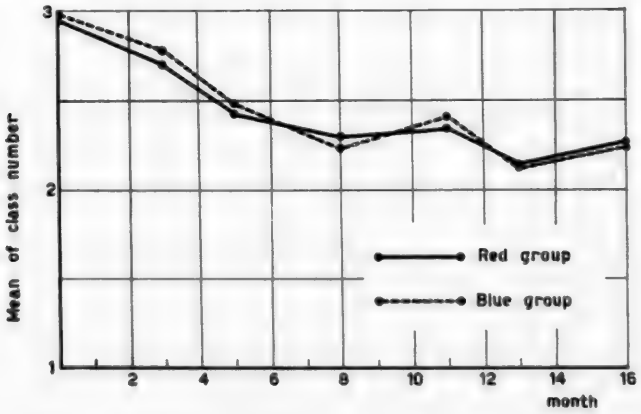

Fig. 3.— New caries per hundred sound surfaces (Table II).

TABLE II. NEW CARIES IN SOUND SURFACES

	RIGHT MAXILLA									T_8S LEFT MAX.	T_{16} MAX.	T_{16} MAND.	T_{32} MAX.+ MAND.
	M_2		M_1		P_2		P_1						
	D	M	D	M	D	M	D	M	T_8D				
						Red							
Sound surfaces	12	37	54	13	21	39	48	38	261	251	512	449	961
Caries	1	16	21	7	12	19	21	7	106	85	191	186	377
Per cent caries	12	43	39	54	60	49	45	20	41	34	37	41	39
						Blue							
Sound surfaces	12	33	47	22	27	36	42	43	262	256	518	441	959
Caries	1	11	23	13	16	13	15	7	100	84	184	193	378
Per cent caries	8	33	50	59	61	36	37	16	38	33	36	44	39

For key to symbols, see Table I.

for each type of surface (Fig. 3) scatter somewhat more than before, but it is evident that no material differences between red and blue groups exist (compare also Table IV).

New Caries in New Surfaces.—The diagnosis "sound surface" in a roentgenologic study like the one at hand depends of course on projection. Small lesions not on the apex of the approximal curve might be missed. So it remains uncertain whether new caries in sound surfaces is indeed new caries. One might postulate, however, that the surfaces of elements erupting during the experimental period (P_2, M_2, and P_1) were completely sound. The number of these surfaces is not large but it seemed worth while to calculate the number of cavities, which were formed in these surfaces during the experimental period. Table III gives the results. Though the data scatter for each type of surface due to smaller numbers the totals are quite equal for the blue and red groups.

TABLE III. NEW CARIES IN NEW SURFACES

	RIGHT MAXILLA									T_8S LEFT MAX.	T_{16} MAX.	T_{16} MAND.	T_{32} MAX.+ MAND.
	M_2		M_1		P_2		P_1						
	D	M	D	M	D	M	D	M	T_8D				
						Red							
New surfaces	10	14	0	0	16	14	7	6	67	71	138	98	236
Caries	2	1	–	–	5	1	0	0	10	13	23	22	46
Per cent caries	25	11	–	–	34	7	0	0	16	18	17	23	19
						Blue							
New surfaces	7	22	0	0	10	10	5	1	55	58	113	143	256
Caries	0	2	–	–	4	1	1	0	9	8	17	30	47
Per cent caries	0	9	–	–	45	15	20	0	16	14	15	21	18

For key to symbols, see Table I.

Lactobacilli.—Lactobacilli counts were made of all patients at bi- or trimonthly intervals during the experimental period. A study of the resulting material arranged in various ways did not show a difference between red and blue groups. To present all our data in a concise form the following method was chosen:

Every time a lactobacillus count was taken the boys were divided in Classes I to V according to the number of lactobacilli in their saliva. A frequency diagram of each series of counts was then made and the mean calcu-

lated by giving to each class the numerical value (1 to 5) of the class number (I to V). A mean of 2.96 thus indicates that the mean is between Classes II and III but much nearer Class III (in fact, just to the left side of the middle of Class III). The mean of the logarithm of the number of lactobacilli would have filled the purpose equally well, but the chosen method has the advantage of simpler calculation, the results being nearly equal. In Fig. 4 these means

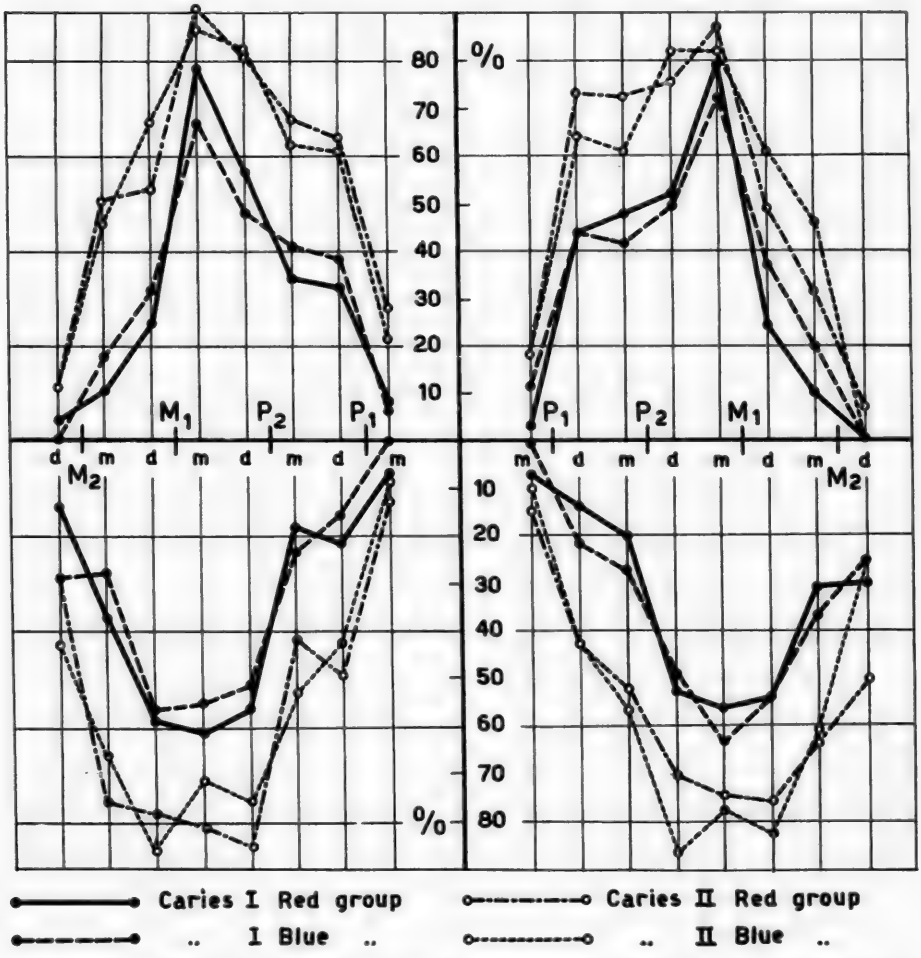

Fig. 4.—Mean number of lactobacilli (mean of class number) during the experimental period.

are plotted against time for red and blue groups separately. During the first 6 months of the experiment there is a significant decrease of the mean number of lactobacilli which might be ascribed to a general increase of mouth hygiene under the influence of the experiment, or to a seasonal influence, or to any cause one would like to invent. However this might be, it is evident that there is no significant difference between blue and red groups.

DISCUSSION

The reproducibility of caries evaluation with our method was again confirmed (Table I). Caries percentages at the start and at the end of the experiment are indeed remarkably uniform in red and blue groups not only for total numbers but also for each type of surface (Fig. 1). Caries increase was equal in both groups (Fig. 2) and comparable to results in earlier experiments.[2] The results are given in summary form in Table IV. Neither the total material (Table IV, *A*) nor the new caries in sound surfaces (*B*) or the new caries in surfaces which erupted during the experimental period (*C*) shows any significant difference between red and blue groups. The experiment shows beyond doubt that, in a tooth paste of the composition used, ammonium phosphate (5 per cent) and urea (3 per cent) have no influence on the incidence of approximal caries in the premolar and molar regions for boys between 10 and 14 years. Occlusal caries and approximal caries in anterior teeth were not studied. The mean number of lactobacilli decreased somewhat during the experiment but equally so in both groups. The use of the ammoniated tooth paste did not reduce the (mean) number of lactobacilli to a lower level than in the control group (Fig. 4).

TABLE IV. SUMMARY OF RESULTS

	I			II		INCREASE	
	SURFACES	CARIES	PER CENT	CARIES	PER CENT	CARIES	PER CENT
A. *Caries Incidence at Start (I) and at End (II) of Experimental Period*							
Red	1,642	642	39.0	1,032	62.8	390	23.8
Blue	1,676	667	39.8	1,063.5	63.4	396.5	23.6
B. *New Caries in Sound Surfaces*							
Red	961			377.5	39.2		
Blue	959			378	39.4		
C. *New Caries in New Surfaces*							
Red	236			46	19.5		
Blue	256			47	18.4		

SUMMARY

Caries was studied in 166 boys of 10 to 14 years, living in two boarding schools. The boys were carefully divided in two groups with about the same dental history and caries incidence. One group brushed their teeth with an ammoniated dentifrice (5 per cent ammonium phosphate, 3 per cent urea), the other group with a control paste.

Proximal caries in molars and premolars was evaluated from duplicate roentgenograms at the beginning and at the end of the experimental period with a method described previously. The standard error was quite small (0.6 per cent). Table IV summarizes the results. The caries increase after one and one-half years was remarkably equal in both groups.

The use of the ammoniated tooth paste had no effect on caries incidence or on the number of lactobacilli in the saliva (Fig. 4).

The equality of the caries increment in both groups shows the advantages of a careful selection of the experimental and control group and confirms the reproducibility of the used method of caries evaluation.

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THE RELATION OF CARIES ACTIVITY TO LACTOBACILLUS COUNTS AND TYPES AND TO THE FLUORIDE CONTENT OF DRINKING WATER

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PAST investigations, such as those of Becks[1] and others, reviewed by Rosebury[2] and Shaw,[3] have shown a correlation of numbers of lactobacilli in the saliva with the extent of dental caries. However, this correlation is not found in every individual, and the validity of the count as an index to caries activity has been questioned by Boyd, Cheyne, and Wessels[4] and Glass.[5]

That there are antigenic and fermentative differences in oral lactobacilli, including differences in ability to produce acid from glucose, has been amply demonstrated.[6-11] It is quite possible that a specific type of lactobacillus might be more capable of inciting caries than others, and would, therefore, be active in smaller numbers. This might explain some of the lack of correlation observed between counts and caries activity. That such a possibility may exist was shown in an investigation of a small group of varied ages, in our laboratory three years ago.[12] It was the purpose of this study to investigate further the relationship of numbers and types of lactobacilli to dental caries in a limited age group of children, and to determine whether fluoride in the drinking water has any effect on the type of lactobacilli found in the saliva.

EXPERIMENTAL

Three hundred eighty-three school children, aged 11 to 14 years, who had resided continously in the areas chosen, were examined with mirror and explorer by one of us (R.A.D.) for decayed, missing, and filled teeth, and decayed and filled surfaces. One hundred ninety-seven were students enrolled in Skinner Junior High School, Denver, Colo., where the drinking water contains 1 ppm fluoride, and 186 were students in Boulder, Colo., where there is no fluoride in the water.

Lactobacillus counts were determined by Hadley's method.[13] Twenty milliliters of saliva, stimulated by chewing paraffin, was collected between 10 and 11 A.M. from each individual. Samples were taken on two consecutive days. To determine the predominating type of lactobacillus, two colonies were picked at random from each plate to peptonized milk agar. When growth occurred, these strains were inoculated into broth containing 1 per cent salicin, raffinose, rhamnose, mannitol, or sorbose. They were also inoculated into glucose broth and the pH was determined after four days' incubation at 37° C. If no organism was found on the tomato juice agar, a plate was streaked again from the dextrose

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broth in which the original saliva dilution had been made. This broth had been incubated at 37° C. Colony morphology was studied with a 7X wide field microscope, which was also used in making the counts. Microscopic morphology was studied on each strain. All gram-positive rods growing on the pH 5 tomato juice agar were considered to be lactobacilli.

Because mold contamination was sometimes troublesome, sodium azide was included in the tomato juice agar on some of the later counts.[14] When first used, 99 individuals' saliva was plated out, both with and without azide. There was no significantly great difference in the counts on the two plates, and while molds occasionally overgrew those without azide, they were never found in the others. Three consecutive counts were performed on some of the first samples taken, but it was found that the counts and fermentative types did not vary enough to justify taking more than two samples for this kind of an investigation. Finally, two colonies were picked from each plate to determine the predominating fermentative type. Using twenty-three different saliva samples, and picking six to ten colonies from each plate, the same fermentative type was picked from a given plate 91 per cent of the time. Selecting two colonies from each plate would make the chances of getting the predominating type even better than this.

Dental and saliva examinations were performed in the same way on most of these children one year later.

RESULTS AND DISCUSSION

Relation of Fluoride Content of Water to Caries Activity.—The extent of caries in the Boulder group with no fluoride was considerably greater than in the Denver group with 1 ppm fluoride in the water. The differences in decayed, missing, and filled teeth and surfaces is shown in Table I. It was also found that 23 per cent of the Denver group showed no evidence of past or present caries, while only 3 per cent of the Boulder group were completely free. The new lesions found one year after the first examinations are given in Table II. There was an increase in both groups, but a much greater increase in the Boulder group with no fluoride in the water.

Colonial, Microscopic, and Fermentative Types.—The commonest colony type found was large, white, convex and smooth. Microscopically, the organism was usually a thick rod with blunt ends, and often in chains. However, the microscopic appearance of the organisms from this colony type, as well as the

TABLE I

EXTENT OF CARIES IN THE DENVER AND BOULDER GROUPS

SCHOOL	DENVER	BOULDER
FLUORIDE CONTENT OF WATER	1 ppm	0 ppm
Number examined	197	186
Average number of		
Decayed teeth	.46	.90
Decayed and filled teeth	3.10	6.50
Decayed, filled, and missing teeth	3.15	7.06
Decayed surfaces	.49	.99
Decayed and filled surfaces	3.40	9.70

TABLE II

AVERAGE NUMBER OF NEW LESIONS AFTER ONE YEAR

SCHOOL	DENVER	BOULDER
FLUORIDE CONTENT OF WATER	1 ppm	0 ppm
Number examined	159	164
Average number of		
New decayed teeth	.66	1.96
New decayed surfaces	1.02	3.65
Per cent with new lesions	37.1	80.0

others, often varied. The next most common was a flat rough type. The rough colony appeared more often in the Boulder group than in the Denver group. There did not appear to be any correlation between colony and microscopic appearance and fermentation reactions. Furthermore, the appearance of the colony was found to change upon subsequent transfer. For these reasons only fermentative type and counts were studied in relation to caries activity.

The lactobacilli were classified into types according to the scheme given in Table III.[12]

TABLE III

TYPE I	TYPE II	TYPE III
pH below 5	pH below 5	pH 5 or above
Salicin +	Salicin +	Salicin −
Rhamnose +	Rhamnose −	Rhamnose −
Mannitol +	Mannitol +	Mannitol −
Raffinose − usually	Raffinose − usually	Raffinose + usually

A study of the fermentation of sorbose was included because Orland[11] reported an antigenic group of lactobacilli, which were characterized by their ability to ferment rhamnose and sorbose.

Distribution of Fermentative Types in the Two Areas.—The data in Table IV make it evident that the prescence or absence of 1 ppm fluoride in the water bore but little relation to the proportion of those children who have rhamnose-fermenting, or Type I, II, or III lactobacilli, in their saliva. There were about 15 per cent more children with sorbose-fermenting lactobacilli in the Boulder group. It is possible that larger amounts of fluoride would accentuate these small differences and indicate some influence of fluoride on the flora. It has been previously shown in vitro[15] that lactobacilli, grown in a medium containing 100 ppm of fluoride, lost their acidogenic properties after several months.

TABLE IV

DISTRIBUTION OF FERMENTATIVE TYPES

TYPE OF LACTOBACILLUS PREDOMINATING IN SALIVA	PER CENT WITH FERMENTATIVE TYPE	
	DENVER	BOULDER
Rhamnose +	38.1	43.0
Sorbose +	42.2	57.0
Type I	40.1	42.0
Type II	27.9	25.5
Type III	16.7	10.6
Number examined	197	188

This did not occur when they were grown in 1 ppm fluoride. It was thought that prolonged contact with 1 ppm, over several years, as would occur when water contains this amount, might bring about a predominance of poor acid-producing lactobacilli in the saliva. From this study it would seem that 1 ppm fluoride has little influence on the type which is found. It might be of some interest to note that the per cent of Type I lactobacillus found in the age group studied in this investigation was considerably higher, and Type II lower, than that previously found[12] in a predominantly older group. It seems possible that this might be an explanation of the decrease in caries activity that is often seen as an individual grows older.

Correlation Between Counts and the Presence of Fluoride.—That there is a greater per cent of the children in the Boulder area having lactobacillus counts of over 10,000 per ml., and a smaller per cent having counts between 50 and 9,999 per ml., is shown in Table V. These values are very similar to those discussed by Dean, Jay, Arnold, McClure, and Elvove.[16] There were, however, the same percentage in each group having negative counts.

TABLE V

PER CENT OF CHILDREN HAVING NEGATIVE, LOW AND HIGH LACTOBACILLUS COUNTS

		NUMBER OF LACTOBACILLI PER ML. OF SALIVA					
		0		50 to 9,999		10,000 or more	
	TOTAL NUMBER	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
Boulder	188	31	16	32	17	125	67
Denver	196	31	16	69	35	96	49

Correlation Between Caries Incidence, Lactobacillus Counts, and Lactobacillus Types.—The mean values for the extent of caries in both the Denver and Boulder groups of those exhibiting a certain type of lactobacillus, and of those with high and low lactobacillus counts, are shown in Tables VI and VII. Present caries experience, as measured by decayed teeth or decayed surfaces, was found to be more highly correlated with the fermentative type of lactobacillus, and also with counts than with total past and present experience. This

TABLE VI

RELATION OF NUMBER AND FERMENTATIVE TYPE OF LACTOBACILLI TO CARIES (BOULDER)

SALIVA ANALYSIS	NUMBER EXAMINED	AVERAGE NUMBER OF			
		D	DF	DS	DFS
No lactobacillus isolated	24	.04	4.3	0.4	6.8
Less than 10,000 lacto per ml.	63	.49	5.2	.50	7.4
10,000 or more lacto per ml.	123	1.11	7.0	1.20	11.0
Type I predominating in saliva	74	1.10	6.6	1.20	10.2
Type II predominating in saliva	47	.61	6.7	.70	10.7
Type III predominating in saliva	16	.62	6.6	.50	9.9
Rhamnose fermenters predominating in saliva	78	1.10	6.5	1.20	9.9
Sorbose fermenters predominating in saliva	105	1.00	6.5	1.10	10.0

D, decayed teeth; DF, decayed and filled teeth; DS, decayed surfaces; DFS, decayed and filled surfaces.

TABLE VII

RELATION OF NUMBER AND FERMENTATIVE TYPE OF LACTOBACILLI TO CARIES (DENVER)

SALIVA ANALYSIS	NUMBER EXAMINED	AVERAGE NUMBER OF D	DF	DS	DFS
No lactobacillus isolated	17	.06	1.0	.06	1.05
Less than 10,000 lacto per ml.	100	.35	2.58	.40	3.36
10,000 or more lacto per ml.	95	.59	3.50	.59	4.50
Type I predominating in saliva	79	.57	3.30	.63	4.10
Type II predominating in saliva	55	.34	3.00	.38	3.60
Type III predominating in saliva	33	.42	3.50	.48	4.90
Rhamnose fermenters predominating in saliva	80	.56	3.30	.60	4.20
Sorbose fermenters predominating in saliva	96	.55	3.40	.57	4.08

D, decayed teeth; DF, decayed and filled teeth; DS, decayed surfaces; DFS, decayed and filled surfaces.

might be expected, since the lactobacillus count made at the time of examination was used, and any flora present at this time would be more likely to influence the condition of the teeth now than in the past, when the flora might have been quite different.

In both areas the average number of decayed teeth and decayed surfaces in the group having Type I lactobacilli is higher than in those having the other types. These values are very nearly the same as the mean values for those having over 10,000 lactobacilli per milliliter, and for those in which rhamnose fermentation is taken as the only criterion for classification. The values are but slightly lower for the group having sorbose-fermenting lactobacilli predominating in the saliva.

Correlation Between Caries Activity and All Factors.—Since the best correlation was obtained between the number of decayed surfaces and the type

TABLE VIII

RELATION OF DECAYED SURFACES TO LACTOBACILLUS COUNTS AND TYPES, AND TO FLUORIDE CONTENT OF WATER

FLUORIDE CONCENTRATION	TYPE OF LACTOBACILLI IN SALIVA: TYPE I COUNT UNDER 10,000	TYPE I COUNT 10,000 AND OVER	TYPE I ALL COUNTS	TYPES II AND III COUNT UNDER 10,000	TYPES II AND III COUNT 10,000 AND OVER	TYPES II AND III ALL COUNTS	ALL TYPES COUNT UNDER 10,000	ALL TYPES COUNT 10,000 AND OVER	ALL TYPES ALL COUNTS
0 ppm									
No. of D. S.	21	72	93	8	46	54	29	118	147
No. examined	16	59	75	21	48	69	37	107	144
Mean	1.31	1.22	1.24	0.38	0.95	0.78	0.78	1.10	1.02
S.E.M.	±0.44	±0.19	±0.17	±0.24	±0.22	±0.17	±0.24	±0.14	±0.12
1 ppm									
No. of D. S.	12	36	48	20	16	36	32	52	84
No. examined	35	45	80	45	44	89	80	89	169
Mean	0.34	0.80	0.60	0.44	0.36	0.40	0.40	0.58	0.50
S.E.M.	±0.14	±0.21	±0.13	±0.22	±0.09	±0.09	±0.11	±0.12	±0.08
Totals									
No. of D. S.	33	108	141	28	62	90	61	170	231
No. examined	51	104	155	66	92	158	117	196	313
Mean	0.65	1.04	0.91	0.42	0.67	0.57	0.52	0.87	0.74
S.E.M.	±0.18	±0.14	±0.10	±0.13	±0.13	±0.09	±0.11	±0.10	±0.07

0 counts with no organisms isolated were not included.
D.S., decayed surfaces.

of lactobacillus found, as determined by the criteria just given, the data in Table VIII were compiled. This attempts to bring out possible interrelationships between types, counts, and the fluoride content of the drinking water. It is again apparent from these data that in both Boulder and Denver those children with Type I lactobacilli in their saliva have a higher average number of decayed surfaces than those with Types II and III. It also shows that those with 10,000 or more lactobacilli have more decayed surfaces than those with less than 10,000 lactobacilli per milliliter of saliva. Furthermore, it appears that those with a low count and a Type I in Boulder, where there is no fluoride in the water, have a much higher number of decayed surfaces (1.31) than those with a low count and Type II or III lactobacillus (.38). Those with a high count and Type I have a slightly higher number of decayed surfaces (1.22) than those with a high count and Type II or III (.95). In Denver those with counts under 10,000 and Type I differ little from those with Types II and III, but those with high counts and Type I have a higher number of decayed surfaces (.8) than those with Types II and III (.36). However, a statistical analysis of these data, using the standard error of the means to determine the critical ratio, shows the values to be without significance. This may be because of small numbers in some of the groups, or because of the wide variation in the number of decayed surfaces per child.

The per cent of those with caries is compared in all of the groups in Table IX. This, of course, ignores differences in the extent of caries in different children in the carious group. However, the same trends were shown as found in Table VIII, and with these data it is possible to show statistically significant

TABLE IX

RELATION OF PRESENCE OF CARIES TO LACTOBACILLUS COUNTS AND TYPES, AND TO FLUORIDE CONTENT OF WATER

FLUORIDE CONCENTRATION	TYPE OF LACTOBACILLI IN SALIVA								
	TYPE I			TYPES II AND III			ALL TYPES		
	COUNT UNDER 10,000	COUNT 10,000 AND OVER	ALL COUNTS	COUNT UNDER 10,000	COUNT 10,000 AND OVER	ALL COUNTS	COUNT UNDER 10,000	COUNT 10,000 AND OVER	ALL COUNTS
0 ppm									
No. with caries	8	33	41	3	20	23	11	53	64
No. examined	16	59	75	21	48	69	37	107	144
% with caries	50	56	55	14	42	33	30	50	44
S.E. %	±12.8	±6.5	±5.8	±7.7	±7.2	±5.7	±7.6	±4.9	±4.1
1 ppm									
No. with caries	6	16	22	9	14	23	15	30	45
No. examined	35	45	80	45	44	89	80	89	169
% with caries	17	36	28	20	32	26	19	34	27
S.E. %	±6.4	±7.2	±5.0	±6.0	±7.1	±4.7	±4.4	±5.0	±3.4
Totals									
No. with caries	14	49	63	12	34	46	26	83	109
No. examined	51	104	155	66	92	158	117	196	313
% with caries	27	47	41	18	37	29	22	42	35
S.E. %	±6.3	±4.8	±4.0	±4.8	±5.0	±3.6	±3.8	±3.5	±2.7

differences in the values from the Boulder group. In this group of those who had low counts and Type I lactobacilli in the saliva, 50 per cent had caries, as compared to 14 per cent with caries in those with Type II or III. There were 56 per cent with caries in those with high counts and Type I, compared to 42 per cent with caries in those with high counts and Type II or III. In the Denver group there was a slightly higher (not statistically significant) per cent with caries (36 per cent) in those with a high count and Type I lactobacilli, compared to 32 per cent in those with a high count and Type II or III. The same trends were found when the per cent of total carious teeth in each of the groups was compared. These findings may indicate the following: (1) That types of lactobacilli and counts are related to caries only when the influence of fluoride on the lactobacillus or on the resistance of the tooth, is absent. In this absence, either a small number of "active" Type I, or a large number of any type, may be found associated with an increase in caries. (2) When fluoride is present to inhibit caries, a large number of Type I lactobacilli are required before any increase in decayed surfaces is found.

Preliminary studies on the rate of acid production from glucose, as measured by titrations with growing cells and manometric measurements of glycolysis with resting cells, indicate Type I to be more active than Type II or III.

In a subsequent paper the relation of counts and types to the caries activity in the following year, and the changes in counts and types after a year's time, in the same individuals, will be reported.

SUMMARY

1. The incidence of caries and the attack rate the following year were much higher in the Boulder group where no fluoride was present in the water than in the Denver group where there was 1 ppm fluoride.

2. There does not appear to be any appreciable difference in the relative proportion of any given fermentative type of lactobacillus in the area with fluoride and the area without fluoride.

3. The incidence of caries was higher in children who had high lactobacillus counts than in those with low counts, and higher in those with Type I lactobacilli in their saliva than in those with other types.

4. (a) In the children whose drinking water contained no fluoride, the number of carious surfaces and the per cent of those with caries, was much higher in the group with low lactobacillus counts when Type I was present. These values were higher, but less so, in the group with high counts when Type I was present.

(b) In the children whose drinking water contained 1 ppm fluoride, the number of carious surfaces and the percentage of those with caries was slightly higher only in the group with high lactobacillus counts when Type I was present.

5. The possible significance of these findings was discussed.

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STUDY OF EFFECT ON TEETH OF INTERMITTENT FLUORIDATION OF A COMMUNITY WATER SUPPLY

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THE city of Pulaski in Giles County, Tenn., affords an area in which the intermittent fluoridation of water can be studied. The effect on the teeth of children of intermittent fluoridation of a municipal water supply has not been reported.

The Pulaski water supply is derived from Richland Creek, while the surrounding area is served by ground water. The intermittent fluoridation of the water supply of Pulaski possibly is related to the water saturation in the ground. Alexander[1] suggested that when the ground is completely saturated with water there is less passage of water over the phosphate rock and therefore less solution of fluorides. The fluoride content of Pulaski water varies from 0.2 to 1.3 ppm. The ground waters of the adjacent study area in the county have unknown fluoride values.

The condition of the teeth of the children using the municipal water supply of Pulaski (0.2 to 1.3 ppm. F.) has been compared with the condition of the teeth of the children living outside of the city using ground water (wells or springs) of unknown fluoride content, to determine whether a correlation exists between the surface water supplies of known high intermittent fluoride content and the ground water supplies.

Dean,[11] Arnold,[3] Klein,[17] and many other investigators have shown that groups of children who consume waters containing small amounts of fluorine (0.5 ppm. or more) experience less dental decay than do comparable groups of children who use fluoride-free water. Arnold[3] has shown that children who are born and reared in communities whose domestic water supplies contain more than 0.5 ppm. fluorine experience less dental caries than do children living in areas where the domestic water supply is fluoride-free. Arnold[2] has proposed that water containing 0.5 to 0.7 ppm. fluorine in the southwestern United States might have an effect equal to that of water of 1.0 to 1.5 ppm. fluoride in the north central section, and concluded that only a small amount of fluorine is necessary to produce the desired effects.

McKay,[33] Churchill,[30] Smith,[34] and Dean[6, 7, 31] have demonstrated that small quantities of fluorine consumed in a water supply during tooth development may result in mottled enamel, 1 ppm. representing a safe threshold.[31]

Russell[19] has reported a study of a community in which the water supply was accidentally fluoridated to a mean level of 1.15 ppm. fluorine for eighteen months. On the basis of caries prevalence, he found no demonstrable inhibition of dental caries in the younger children of the community. In the same group he did find that 1 per cent of the girls and 5 per cent of the boys had

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very mild or mild dental fluorosis. Klein,[15] in an investigation of a community water supply that was accidentally fluoridated (1.2 to 2.2 ppm. fluorine), found a marked reduction in the incidence and prevalence of dental decay in persons born in or migrating to the area. He concluded that the deciduous teeth were protected to a greater extent against caries in those children to whom the fluoride city water was available than among those children who used farm well water, even though their homes were in the same area. Children born, reared, and residing in the fluoride area who had consumed the city water exhibited lower DMF rates at every age than did children who consumed the city water in the nonfluoride area.

Hardgrove and Bull[13] have established a "def" rate of 1.27 for children 5 to 6 years of age who consumed water with a fluoride content of 2.3 ppm. A group that consumed water containing .05 ppm. had a def rate of 4.82. Bull[5] claimed that the def rate for deciduous teeth increases when the fluorine content falls below 2 ppm. while the DMF rate remains as low at 2 ppm. as it does at 1 ppm.

Velu[28] reported observations which show that mottled enamel exists in many phosphate zones of North Africa. He has demonstrated that this dystrophy is only an occasional symptom of chronic intoxication resulting from prolonged consumption of water which has flowed through layers of phosphate.

Dean[8] observed mottled enamel in a few families using ground water which had traversed rock phosphate beds in Hickman and Maury counties of Tennessee. The number of children examined in the areas was few, but the prevalence of mottled enamel was high, suggesting the presence of fluorine in this area. Smith and Whitlatch[22] have classified the three types of phosphate rock that occur in Tennessee as brown, blue, and white rock. The brown rock, according to Alexander,[1] contains the highest amount of phosphate and fluoride as F. The principal sources of the brown rock deposits are the Hermitage, Bigby, Cannon, and Leipers formations.[23] The chemical composition of the Bigby layer of phosphate rock shows the fluoride content as F to be 2 per cent.[24] The phosphate areas around Pulaski, Tenn., are the Bigby and Cannon layers of the brown rock containing as much as 3 per cent fluoride as F.[25]

METHOD

Mouth mirror and explorer examinations were made of the teeth of white children 6 to 17 years of age in Pulaski elementary and high schools. Similar examinations of the same age groups were completed in Fayetteville, Tenn., elementary and high schools. The Fayetteville children were used as a control group. The water supply for Fayetteville comes from springs in the highland rim of Tennessee and is constantly fluoride-free. We completed all examinations using the same methods. The caries diagnosis utilized by each of us was standardized.

The information concerning the source of water and the period of use of a particular water was obtained from each child by the schoolteachers from school records and questioning of the child. The dentists checked the teachers' information by questioning each child during the examination.

Table 1 presents the decayed, missing, and filled permanent teeth (DMF) per child in Pulaski and Fayetteville. It will be noted that there is a lower DMF per child at each age group of the Pulaski children when compared with the Fayetteville children. Statistical analysis reveals that, with the exception of the 6 year age group, all differences in the DMF rates per child are significant.

TABLE I. DECAYED, MISSING, AND FILLED PERMANENT TEETH PER CHILD (DMF PER CHILD) IN PULASKI AND FAYETTEVILLE IN 1948

AGE (YR.)	FAYETTEVILLE			PULASKI		
	NO. OF CHILDREN	TOTAL NO. DMF TEETH	DMF TEETH PER CHILD	NO. OF CHILDREN	TOTAL NO. DMF TEETH	DMF TEETH PER CHILD
6	47	30	.64 ± .14	87	44	.51 ± .51
7	43	101	2.34 ± .23	105	65	.62 ± .11
8	43	127	2.95 ± .25	72	75	1.04 ± .17
9	56	251	4.48 ± .40	75	140	1.87 ± .23
10	46	226	4.91 ± .53	86	180	2.09 ± .23
11	57	388	6.81 ± .61	78	177	2.27 ± .23
12	48	344	7.17 ± .72	75	251	3.35 ± .40
13	60	555	9.25 ± .56	106	426	4.02 ± .34
14	98	945	9.64 ± .53	91	407	4.47 ± .53
15	27	319	11.81 ± 1.48	79	395	5.00 ± .43
16	13	120	9.23 ± 1.45	32	220	6.88 ± .81
17	3	35	11.67 ± .33	8	50	6.25 ± 1.84
6-17	541	3441	6.35 ± .22	894	2430	2.72 ± .01

Table II presents the data on children in Pulaski and Fayetteville who have no DMF teeth. When age groups are compared there is a marked difference in the percentage of children with DMF teeth, but since the numbers are small in the Fayetteville group, no groups are statistically significant. There is a significant difference in the percentage of all the children in Pulaski who have no DMF teeth compared with all those from Fayetteville.

Table III presents comparisons of the def rates per child of the Fayetteville and Pulaski children. Although the def rates are lower for each age

TABLE II. CHILDREN IN PULASKI AND FAYETTEVILLE HAVING NO DMF TEETH IN 1948

AGE (YR.)	FAYETTEVILLE			PULASKI		
	NO. OF CHILDREN	NO. OF CHILDREN WITH NO DMF TEETH	PER CENT OF CHILDREN WITH NO DMF TEETH	NO. OF CHILDREN	NO. OF CHILDREN WITH NO DMF TEETH	PER CENT OF CHILDREN WITH NO DMF TEETH
6	47	32	68.09 ± 6.8	87	69	79.31 ± 4.3
7	43	7	16.28 ± 5.6	105	72	68.57 ± 4.5
8	43	5	11.63 ± 4.9	72	36	50.0 ± 5.8
9	56	8	14.29 ± 4.6	75	26	34.67 ± 5.4
10	46	1	2.17 ± 2.2	86	26	30.23 ± 4.9
11	57	3	5.26 ± 2.9	78	18	23.77 ± 4.8
12	48	1	2.08 ± 2.05	75	19	25.33 ± 5.01
13	60	1	1.67 ± 1.6	106	19	17.92 ± 3.7
14	98	3	3.06 ± 1.7	91	14	15.38 ± 3.78
15	27	0	0	79	13	16.45 ± 4.1
16	13	0	0	32	3	9.38 ± 5.1
17	3	0	0	8	1	12.50 ± 11.67
6-17	541	61	11.25 ± 1.4	894	316	35.35 ± 1.6

TABLE III. DECAYED, FILLED, AND DECIDUOUS TEETH INDICATED FOR EXTRACTION (def) CHILD IN FAYETTEVILLE AND PULASKI IN 1948

AGE (YR.)	FAYETTEVILLE			PULASKI		
	NO. OF CHILDREN	NO. DEF TEETH	DEF TEETH PER CHILD	NO. OF CHILDREN	NO. DEF TEETH	DEF TEETH PER CHILD
6	47	374	7.96 ± .74	87	357	4.10 ± .44
7	43	316	7.35 ± .70	105	393	3.74 ± .38
8	43	270	6.28 ± .53	72	249	3.46 ± .43
9	56	286	5.11 ± .52	75	267	3.56 ± .40
10	46	162	3.52 ± .51	86	293	3.41 ± .28
11	57	85	1.49 ± .37	78	94	1.21 ± .28
12	48	25	.52 ± .18	75	26	.35 ± .10
13	60	12	.20 ± .07	106	18	.17 ± .06
6-8	133	960	7.21 ± .39	264	999	3.77 ± .24
6-13	400	1530	3.83 ± .22	684	1697	2.48 ± .12

TABLE IV

PREVALENCE OF DECAYED, MISSING, AND FILLED (DMF) TEETH PER CHILD IN PULASKI WITH REFERENCE TO SOURCE OF WATER AND PERIOD OF USE IN 1948

AGE (YR.)	CHILD. USING MUNICIPAL WATER SUPPLY IN 1948 (MIXED WATER HISTORIES FOR MUNICIPAL WATER SUPPLY)			CHILD. USING WELL OR SPRING WATER IN 1948 (MIXED WATER HISTORIES FOR WELLS OR SPRINGS)			CHILD. USING MUNICIPAL WATER CONT. SINCE BIRTH (PURE WATER HISTORIES FOR MUNICIPAL WATER SUPPLY)			CHILD. USING WELL OR SPRING WATER CONT. SINCE BIRTH (PURE WATER HISTORIES FOR WELLS OR SPRINGS)		
	NO. OF CHILD.	NO. OF DMF TEETH	NO. OF DMF TEETH PER CHILD	NO. OF CHILD.	NO. OF DMF TEETH	NO. OF DMF TEETH PER CHILD	NO. OF CHILD.	NO. OF DMF TEETH	NO. OF DMF TEETH PER CHILD	NO. OF CHILD.	NO. OF DMF TEETH	NO. OF DMF TEETH PER CHILD
6	54	35	.65 ±.21	33	9	.27 ±.16	33	28	.85 ±.32	26	5	.19 ±.69
7	74	51	.69 ±.14	31	14	.45 ±.15	54	41	.76 ±.18	25	14	.56 ±.18
8	47	44	.94 ±.18	25	31	1.24 ±.32	33	31	.94 ±.20	16	22	1.38 ±.41
9	46	79	1.71 ±.22	29	61	2.10 ±.49	31	50	1.61 ±2.35	23	42	1.83 ±.48
10	56	112	2.00 ±.24	30	68	2.27 ±.46	21	27	1.29 ±.33	19	53	2.79 ±.65
11	48	76	1.58 ±.23	30	101	3.37 ±.48	28	43	1.54 ±.30	15	49	3.27 ±.72
12	47	148	3.15 ±.48	28	103	3.68 ±.62	26	38	1.46 ±.34	22	75	3.41 ±.82
13	54	214	3.96 ±.49	52	212	4.08 ±.46	24	76	3.17 ±2.58	33	122	3.70 ±.52
14	49	202	4.12 ±.51	42	205	4.88 ±1.22	11	19	1.73 ±.63	27	106	3.93 ±.74
15	43	183	4.26 ±.54	36	212	5.89 ±.67	16	38	2.38 ±.65	22	126	5.73 ±.85
16	15	126	8.40 ±1.21	17	94	5.53 ±1.02	5	39	7.80 ±1.92	10	51	5.10 ±1.45
17	6	25	4.17 ±1.61	2	25	12.5 ±1.51	2	1	.50 ±.50	0	0	0.00
6-17	539	1295	2.40 ±.13	355	1135	3.20 ±.19	284	431	1.52 ±.12	238	665	2.79 ±.22

group of Pulaski, only the 6 to 8 year age group rate is significantly lower as it is for the 6 to 13 year age group as a whole. The def rate for the 6 to 8 age group appears to be of more significance than the DMF rate since so few permanent teeth have erupted in this period of the mixed dentition.

Table IV presents the data on the prevalence of decayed, missing, and filled permanent teeth in Pulaski in relation to the source of water and the period during which this source of water was used. There is a higher prevalence of DMF teeth for those children with mixed water histories who used well or spring water in 1948, than for those with mixed water histories who used municipal water supplies in 1948 for the age group 6 to 17 years of age. The results indicate that the difference is not consistent in all age groups, since only those for the 17 year age group and for the 6 to 17 age group are significant.

Table IV also shows that there is a slight but significant difference between the children who consumed municipal water continuously since birth and those who had consumed well or spring water continuously since birth. The DMF rate for the 6 to 17 year age group, consuming municipal water continuously since birth, is 1.52 teeth per child, whereas, the children consuming well or spring water continuously since birth had a DMF rate of 2.79 teeth per child. The difference between the children with mixed municipal water histories who had 2.40 DMF teeth per child and the group with pure municipal water histories who had 1.52 DMF teeth per child is significant, but the difference between the children with mixed water histories who consumed well or spring water (3.20 DMF teeth per child) and the children with pure water histories who consumed well or spring water continuously since birth (2.79 DMF teeth per child) is not statistically significant.

Table V relates the def prevalence rates compared to the source of water and the period of use. There is no significant difference in any of the def rates in Pulaski with reference to source of water and period of use.

Table VI presents data comparing the fluorosed permanent teeth in Pulaski with the source of water and the period of use. The difference in the number of fluorosed teeth per child of those using municipal water supply in 1948 (mixed municipal water histories) and of those using wells or springs in 1948 (mixed well or spring water histories) is significant for the 9 year age group, the 12 year age group, and the total age group 6 to 17 years of age, the other age groups not being significantly different. A comparison of the differences in fluorosed teeth per child of those using the municipal water supply continuously since birth (pure water histories of the municipal water supply) and those using wells or springs continuously since birth (pure water histories for wells or springs) indicates that the difference only for the 9 year age group and total group 6 to 17 is significant. The difference between the number of fluorosed teeth per child among those using the municipal water supply in 1948 (mixed histories for municipal water supply) and those using the municipal water supply continuously since birth (pure water histories for the municipal supply) is not significant for any individual age group or the total age group 6 to 17 years of age. The same observation holds true for the differences in

TABLE V

PREVALENCE OF DECAYED, FILLED AND DECIDUOUS TEETH INDICATED FOR EXTRACTION (DEF) WITH REFERENCE TO SOURCE OF WATER AND PERIOD OF USE IN PULASKI IN 1948

	CHILD. USING MUNICIPAL WATER SUPPLY IN 1948 (MIXED WATER HISTORIES FOR MUNICIPAL WATER SUPPLY)			CHILD. USING WELL, SPRING, OR CISTERN WATER IN 1948 (MIXED WATER HISTORIES FOR WELLS OR SPRINGS)			CHILD. USING MUNICIPAL WATER CONT. SINCE BIRTH (PURE WATER HISTORIES FOR MUNICIPAL WATER SUPPLY)			CHILD. USING WELL, SPRING, OR CISTERN WATER CONT. SINCE BIRTH (PURE WATER HISTORIES FOR WELLS OR SPRINGS)		
AGE (YR.)	NO. OF CHILD.	NO. OF (DEF) TEETH	NO. OF (DEF) TEETH PER CHILD	NO. OF CHILD.	NO. OF (DEF) TEETH	NO. OF (DEF) TEETH PER CHILD	NO. OF CHILD.	NO. OF (DEF) TEETH	NO. OF (DEF) TEETH PER CHILD	NO. OF CHILD.	NO. OF (DEF) TEETH	NO. OF (DEF) TEETH PER CHILD
6	54	223	4.13 ±.58	33	134	4.06 ±.68	33	119	3.61 ±.68	26	93	3.58 ±1.81
7	74	255	3.04 ±.43	31	138	4.45 ±.76	54	164	3.04 ±.49	25	117	4.68 ±.90
8	47	165	3.51 ±.57	25	84	3.36 ±.62	33	92	2.79 ±.59	16	44	2.75 ±.65
9	46	186	4.04 ±.51	29	81	2.79 ±.63	31	114	3.68 ±.62	23	64	2.78 ±.94
10	56	120	2.14 ±.36	30	73	2.43 ±.31	21	47	2.24 ±.61	19	49	2.58 ±.63
11	48	49	1.02 ±.22	30	45	1.50 ±.35	28	35	1.25 ±.46	15	21	1.4 ±.61
12	47	18	.38 ±.19	28	8	.29 ±.19	26	12	.46 ±.32	22	7	.32 ±.24
13	54	11	.20 ±.11	52	7	.13 ±.07	24	3	.13 ±.09	33	6	.18 ±.10
6-8	175	643	3.72 ±.30	89	356	4.0 ±.41	120	375	3.13 ±.33	67	254	3.79 ±.78
6-13	426	1027	2.41 ±.16	258	570	2.21 ±.20	250	586	2.34 ±.21	179	401	2.24 ±.34

fluorosed teeth per child in those children using well or spring water in 1948 (mixed water histories for wells or springs) and the children who used well or spring water continuously since birth (pure water histories for wells or springs). No fluorosis was observed in deciduous teeth and no fluorosed teeth were noted in the Fayetteville group. Of the fluorosed teeth noted in the Pulaski examinations, three cases of severe mottling were observed, the remainder being of mild type.

There is a vein of phosphate rock extending through the middle of Tennessee from the northern to the southern boundaries. The water supply of Fayetteville in Lincoln County is a spring in the highland rim. Scattered deposits of phosphate are found in Lincoln County but are not significant to the source of municipal water supply of Fayetteville since the fluoride determination of the water supply of Fayetteville has been and is 0.0 ppm. fluorine.

Richland Creek is the municipal water supply of Pulaski, Tenn., and it is fed by branches of several creeks that flow through the richest layer of phosphate rock. The three richest layers of phosphate rock provide the highest amount of fluoride as F. The three layers are the undifferentiated, the Bigby, and the Hermitage. The creek system of Giles County, which makes up the municipal water supply of Pulaski, is confined to the richest phosphate strata of the county. There is some distribution of phosphate rock in Lincoln County,

TABLE VI

FLUOROSED PERMANENT TEETH IN PULASKI WITH REFERENCE TO SOURCE OF WATER AND PERIOD OF USE IN 1948

AGE (YR.)	CHILD. USING MUNICIPAL WATER SUPPLY IN 1948 (MIXED WATER HISTORIES FOR THE MUNICIPAL WATER SUPPLY)			CHILD. USING WELL, SPRING, OR CISTERN WATER IN 1948 (MIXED WATER HISTORIES FOR WELLS OR SPRING)			CHILD. USING MUNICIPAL WATER SUPPLY CONT. SINCE BIRTH (PURE WATER HISTORIES)			CHILD. USING WELLS, SPRINGS, OR CISTERN CONT. SINCE BIRTH (PURE WATER HISTORIES)		
	NO. OF CHILD.	NO. OF FLU. TEETH	NO. OF FLU. TEETH PER CHILD	NO. OF CHILD.	NO. OF FLU. TEETH	NO. OF FLU. TEETH PER CHILD	NO. OF CHILD.	NO. OF FLU. TEETH	NO. OF FLU. TEETH PER CHILD	NO. OF CHILD.	NO. OF FLU. TEETH	NO. OF FLU. TEETH PER CHILD
6	5	12	2.4 ±.63	1	3	3.0	3	8	2.7 ±1.17	1	3	3.0
7	17	65	3.8 ±.47	3	7	2.3 ±.88	14	56	4.0 ±.50	1	1	1.0
8	21	128	6.1 ±.88	6	33	5.5 ±1.52	19	117	6.2 ±.74	4	21	5.3 ±2.74
9	19	129	6.8 ±1.14	5	8	1.6 ±.40	18	126	7.0 ±1.18	3	6	2.0 ±.56
10	19	170	8.9 ±1.73	11	52	4.7 ±1.48	11	85	7.7 ±2.39	9	41	4.6 ±1.75
11	24	296	12.3 ±1.7	8	53	6.6 ±2.57	19	214	11.3 ±1.70	4	24	6.0 ±3.45
12	24	305	12.7 ±1.58	4	13	3.3 ±.75	19	288	15.2 ±2.31	4	13	3.3 ±.75
13	19	189	9.9 ±1.71	10	63	6.3 ±2.97	12	136	11.3 ±2.86	6	36	6.0 ±.80
14	15	161	10.7 ±2.48	8	46	5.8 ±2.13	6	65	10.8 ±2.15	6	32	5.3 ±2.57
15	4	48	12.0 ±4.59	5	54	10.8 ±4.3	2	19	9.5 ±7.54	4	48	12.0 ±5.49
16	3	31	10.3 ±6.18	1	1	1.0	1	8	8.0	0	0	0.0
17	0	0	0.0	0	0	0.0	0	0	0.0	0	0	0.0
6-17	170	1534	9.02 ±.57	62	333	5.43 ±.81	124	1122	9.04 ±6.7	42	225	5.36 ±1.02

but there is very little of the rich Bigby formation found in that county and none is found close to Fayetteville, Tenn., which has a spring as the source of its municipal water supply. The community of Wales, just north of Pulaski on Richland Creek, is the location of a phosphate processing plant which was thought to be the source of the fluoridation of Richland Creek. The three patients with severe cases of mottled enamel detected in the Pulaski examinations came from Wales, which has no municipal water supply.

Tables VII and VIII present the fluorine, in ppm., that is found in the municipal water supplies of Fayetteville and Pulaski distribution systems. The Fayetteville distribution system constantly had 0.0 ppm. fluorine, while the samples of the Pulaski water provided levels that ranged between 0.2 and 1.0 ppm. fluorine.

Fluorine determinations were made on raw untreated water of Richland Creek before it entered the water treatment plant at Pulaski. On Nov. 18, 1948, the water at the bridge and at the plant had 0.5 ppm. of fluorine, while that in the municipal system had 0.20 ppm.

TABLE VII

FLUORINE DETERMINATIONS OF MUNICIPAL WATER SUPPLIES

FAYETTEVILLE SYSTEM		PULASKI SYSTEM	
DATE	FLUORINE PPM.	DATE	FLUORINE PPM.
10/18/48	0.0	8/10/48	0.20
10/20/48	0.0	8/16/48	0.80*
10/26/48	0.0	9/ 2/48	0.70*
10/26/48	0.0	9/14/48	0.80*
11/ 9/48	0.0	9/30/48	1.00*
11/ 9/48	0.0	10/ 6/48	0.50*
11/17/48	0.0	10/19/48	0.50
11/17/48	0.0	11/18/48	0.40
11/17/48	0.0	11/18/48	0.20*

*Duplicate samples analyzed.

TABLE VIII

FLUORINE DETERMINATIONS OF MUNICIPAL WATER SUPPLIES[32]

PULASKI SAMPLES	
DATE	FLUORINE PPM.
4/21/50	1.3
4/22/50	0.3
4/23/50	0.3
4/24/50	0.3
4/25/50	0.4
4/26/50	1.2
4/27/50	1.1

DISCUSSION

The findings indicate that the children of Pulaski have fewer DMF teeth than those of Fayetteville, because they consume a water supply that contains intermittently high amounts of fluorine. Correspondingly, a greater percentage of the children in Pulaski have no DMF teeth. The def rates per child do not agree with those reported by Klein[15] and Bull[5] but do indicate that there is a noticeable effect on the deciduous teeth of the children in Pulaski who consume the water which passes over the phosphate-bearing rock in the Giles County area.

When the Pulaski DMF rates are considered in relation to the source of water and the period of use, it is found that the children using the municipal water supply continuously since birth have a lower DMF rate per child than those using the water from the municipal supply in 1948. There is a lower DMF rate per child for those using the municipal water supply continuously since birth and those using wells or springs continuously since birth, indicating the possibility that the ground water of Giles County in the phosphate rock area is not as high in fluorine content as the surface water. It would have been of interest to compare the fluoride content of well or spring waters and the river or creek water throughout Giles County.

Since the def rates per child of the Pulaski children in relation to source of water and period of use are not statistically significant, the rates will not be discussed nor any conclusions attempted, although they do seem to indicate that the effect of the fluoride water on the deciduous teeth is not as great as that observed on the permanent teeth.

The mottled enamel in all but three children observed in these examinations was of the mild or very mild type. More fluorosed teeth and fewer DMF teeth per child were detected in the children using the municipal water supply with a mixed or continuous exposure to fluoride waters. The difference seems to indicate again that the creek and river waters of the phosphate area in Giles County have a higher fluorine content than do the well or spring waters of this area.

The Giles County area is rich in a phosphate that contains a high fluorine (F) content. The municipal water supply of Pulaski was not accidentally fluoridated, since the results indicate a relative benefit to those children who had used the wells and springs of Giles County. Since the watershed of Richland Creek flows through the richest of phosphate fields it is possible that this creek, as the source of the municipal water for Pulaski, has a greater fluorine content than does the well or spring water of the area because the undifferentiated, the Bigby, and Hermitage layers are the layers richest in phosphate and have the highest fluorine (F) content.

The water samples taken in the Pulaski distribution system are intermittent in F content while the samples taken in Fayetteville are constantly zero. Arnold[3] has suggested that 0.5 ppm. is necessary before appreciably low experience of dental caries can be observed. The data submitted in this paper would seem to indicate that a lessened dental caries can be experienced with an intermittent fluoridation varying from day to day in the fluorine concentration.

SUMMARY

Mouth mirror and explorer examinations were made of the teeth of 894 white children 6 to 17 years of age in the Pulaski elementary and high schools. From Fayetteville schools 542 children served as controls, the municipal water supply of that town containing no trace of fluoride. The dental examinations of the two examiners were similar and standardized, and the impression of the examiners was that the children examined in Pulaski and Fayetteville had similar dietary and economic status.

The data indicate that the children using the water of the Pulaski area have fewer DMF teeth than do the children using the water from the Fayetteville area. The def rate per child of the Pulaski group was found to be 2.48 teeth per child for the 6 to 13 year age group and 3.77 teeth per child in the 6 to 8 year age group. For the Fayetteville group the def rate per child for the 6 to 13 year age group was 3.83, and for the 6 to 8 year age group was 7.21. There was a lower DMF rate per child in those children using the municipal water supply in 1948 with mixed exposure (2.40 for the age group 6 to 17 years of age) and for those using the municipal water supply with a constant exposure (1.52 for the 6 to 17 year age group) than for those using wells or springs with continuous exposure (2.79 for the 6 to 17 year age group).

The municipal water supply of Pulaski, which is Richland Creek, is fed by a system of creeks that flow over and through regions rich in phosphate rock that have a high fluorine (F) content. The intermittent fluoridation of the waters of

Richland Creek is caused by the waters flowing through the areas rich in phosphate and the concentration of the fluoride in the water and appears to be related to the amount of water saturation in the ground.

The results of this study indicate that there is a definite relation between the phosphate rock of the area and the fluoride content found in the ground and surface water of the area. The richer the phosphate rock in this area the higher the fluorine content that can be expected in the water.

CONCLUSIONS

1. The lower DMF and def rates in Pulaski are due to the presence of fluoride in water of that area.

2. The lower DMF rate in the children using the municipal water in 1948 and continuously since birth is due to the presence of a greater fluoride concentration in the creeks of the Pulaski area.

3. The higher prevalence of mottled enamel observed in the Pulaski children who had consumed the municipal water in 1948 or continuously since birth is due to the greater fluoride concentration in the creeks of the Pulaski area.

4. The intermittent fluoridation appears to be due to creeks passing over phosphate rock rich in fluorides at varying rates of speed.

5. There seems to be a definite relation between the phosphate rock of this area and the fluoride content of the water of the same area.

6. A beneficial effect of fluoridation on teeth can be observed when the water supply is intermittently fluoridated in a range of 0.2 to 1.0 ppm.

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THE EFFECTS ON DENTIN OF PROTEOLYTIC AND ACIDOGENIC BACTERIA ISOLATED FROM THE CARIOUS LESION

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INTRODUCTION

PUTREFACTION (proteolysis) of food about the teeth was postulated as a cause of dental caries as early as 1756 by Pfaff.[1] Miller[2] stated that decay of the teeth was designated in the German language as Zahnfaüle (tooth rot) since it was thought to be a putrefactive process. Early American advocates of putrefaction as a cause of caries were Mayr[3, 4, 5] and Stockwell,[6-10] in spite of the fact that neither was able to produce caries in vitro with putrefactive microorganisms. Miller[2] discounted the contentions of Mayr and Stockwell after he failed to produce destruction of the teeth with putrefactive organisms alone. He did find that decalcification by acidogenic bacteria released the organic matrix of dentin for destruction by lytic bacteria. Hence, he believed that proteolytic bacteria played a secondary role in the carious process. Miller's observations were substantiated, in part, by Goadby.[11] Other workers (Gies and Kligler,[12-17] McIntosh, James, and Lazarus-Barlow[18] and Okumura and Nakai[19]) found a variety of microorganisms that could lyse the organic matrix of dentin after its decalcification. Later, Bibby[20] found that certain fungi and actinomycetes could slowly disintegrate sections of intact enamel in vitro, but he found that most, if not all, of the common proteolytic bacteria were ineffectual. Pincus[21, 22] found that the keratinolytic bacteria obtained from various laboratories in New Zealand, Africa, and North America, and that lysed keratins such as wool, hoof, hair, and horn, were unable to lyse the organic matrix of intact enamel. It is apparent that the accumulated evidence has not indicated that proteolytic bacteria alone can attack and lyse intact dentin or enamel, but has indicated that many such bacteria can attack the organic matrix of dentin after its decalcification. In spite of this negative evidence, several groups of investigators (Pincus,[23] Hinds,[24] Gottlieb and Hinds[25] and Frisbie, Nuckolls and Saunders[26]) have postulated that proteolytic bacteria are the initial, if not principal, etiologic factor in dental caries. Since the effects of bacteria and their products upon the intact organic matrices of enamel and dentin have not been resolved, it was felt that further investigations were needed to define the action of various oral bacteria upon these portions of the tooth.

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A diazotization process for determining the accessibility of the organic matrix of dentin has been described elsewhere (Burnett and Scherp[27]). The present paper describes the results of the application of this method to determine the effect upon dentin of various types of proteolytic and acidogenic bacteria isolated from saliva and from the carious lesion. The effects of the proteolytic enzymes, pepsin and trypsin, were also studied.

METHODS

The bacteria utilized in this study have been described in previous publications (Burnett and Scherp[30, 31]). The seventeen strains of lactobacilli represented the types isolated from the superficial carious lesion and from the advancing carious front deep in the dentinal lesion. They were typically homofermentative but were not identified as to species, for many of their biochemical characteristics did not conform to those of any of the recognized species.

The actinomycetes represented a culturally homogeneous, obligatorily anaerobic group isolated from the advancing front of deep dentinal caries. Several of their characteristics did not conform to those of any of the previously described species.

The eight strains of cocci were representative of the group that composed the bulk of the bacterial population of the advancing, deep, dentinal carious lesion, half of which were enterococci, and the other half micrococci. Many of these cocci were caseinolytic but none exerted a discernible proteolytic action on dentinal protein. They were only moderately acidogenic.

The nineteen strains of proteolytic bacteria were typical of a group of 250 strains isolated from saliva and from the superficial layers of dentinal caries. Most of these bacteria were gram-positive, sporulating bacilli. All were able to digest decalcified dentinal protein.

The method of preparing and treating sections of dentin with diazonium salts has been described elsewhere (Burnett and Scherp[27]) and will not be repeated in detail. Diazonium salts will couple to protein to form colored azoproteins if tyrosine and histidine are accessible in the protein chain. Since the presence of such accessible amino acids adjacent to the dipeptide linkage is essential to the action of proteolytic enzymes on these linkages (Bergmann and Fruton[28]), the amount of azoprotein formed is a measure of the amount of protein available for proteolysis and can be determined colorimetrically after alkaline extraction. Similarly, the amount of such azoprotein released into solution by proteolytic enzymes can be used as an index of the rate of proteolysis (Charney and Tomarelli[29]).

Under the conditions described (Burnett and Scherp[27]), dentinal protein, prepared by acid decalcification, formed a constant proportion of azoprotein, with a mean colorimeter reading of 50.2 per milligram dry weight. Deviations from this value indicated alterations in the protein, e.g., the protein from the carious lesion in dentin gave a mean colorimeter reading of 23.3 per milligram dry weight. Similarly, the amount of azoprotein formed from partially decalcified dentin was proportional to the loss in weight, with a

mean colorimeter reading of 18.7 per milligram of weight lost, i.e., dentinal protein was made accessible for reactivity in proportion to decalcification (also see Discussion).

Weighed sections of intact or decalcified dentin were exposed (1) to various strains of the four types of bacteria growing in appropriate broth cultures, (2) to pepsin (Merck, U.S.P.) in several buffered mixtures at pH 2.0, and (3) to trypsin (Pfanstiehl) at pH 8.0. All tests were carried out at 37° C. The exposed sections were then treated by the diazotization method to determine their content of accessible protein.

As an alternative approach to the study of the action of these agents, their effects were determined also on sections of diazotized, decalcified dentin.

RESULTS

The first experiments were directed toward determining the actions, on sections of intact dentin, of the acidogenic and proteolytic bacteria already described. The results of these experiments are summarized in Table I. In testing the action of the lactobacilli, weighed sections of intact dentin were exposed separately to each of seventeen strains growing in 2 per cent glucose broth for twenty-one days. The broth was changed each seven days. The lactobacilli maintained an average pH of 3.41 during twenty-one days. At the end of the test period, the exposed dentin sections had undergone an average weight loss of 74 per cent and gave an average colorimeter reading of 12.9 per milligram of original weight. Their mean colorimeter reading was 18.3 per milligram of weight lost during exposure to the lactobacilli.

TABLE I

THE ACTION OF VARIOUS MICROORGANISMS UPON SECTIONS OF INTACT DENTIN

SPECIES	NUMBER OF STRAINS	pH	PER CENT WEIGHT LOSS	COLORIMETER READING/MG. ORIGINAL WT.	COLOR/MG. WEIGHT LOSS
Lactobacilli	17	3.41	73.9	12.9	18.3
Actinomycetes	10	5.07	15.8	3.7	23.6
Cocci	8	5.62	2.3	1.8	8.4
Proteolytic bacteria	19	7.44	5.2	2.2	49.2

All values are means. Period of incubation was twenty-one days. Actinomycetes in thioglycolate broth; other bacteria in glucose broth. One section was exposed to each strain.

Ten strains of oral filamentous microorganisms (actinomycetes), growing in thioglycolate broth, were allowed to act separately on weighed sections of intact dentin under conditions similar to those of the previous experiment. The actinomycetes maintained an average pH of 5.07, and produced an average weight loss of the dentin sections of 15.8 per cent. These sections had a mean colorimeter reading of 3.7 per milligram of original weight, and an average colorimeter reading of 23.6 per milligram of weight loss. It was found also that intact dentin exposed for four weeks or more to the filamentous microorganisms, growing anaerobically in broth solution, became dark brown. The discoloration of dentin, produced by the filamentous microorganisms, is shown in Fig. 1. This discoloration resembled the dark brown color often seen in

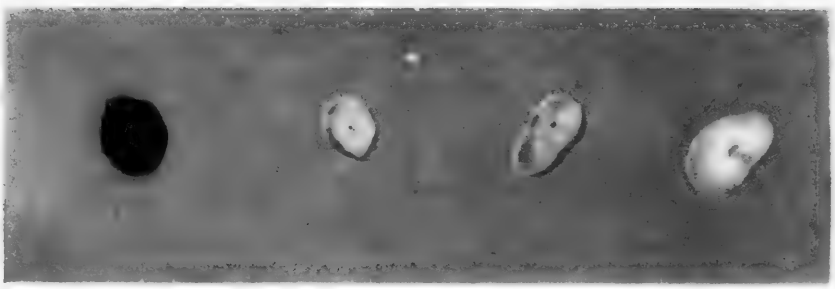

Fig. 1.—A photograph of dentin sections exposed to oral bacteria in broth cultures for twenty-eight days. From left to right the sections had been exposed to actinomycetes, proteolytic bacteria, cocci, and sterile broth.

the dentin of old or arrested caries. The actinomycetes did not produce color changes when such proteins as meat, egg albumin, casein, or gelatin were exposed to them. None of the strains of lactobacilli, cocci, or proteolytic bacteria produced such color changes in dentin under any conditions tested.

Weighed sections of intact dentin were exposed separately to each of eight strains of cocci in 2 per cent glucose broth. The cocci maintained a mean pH of 5.62, which caused an average weight loss of the dentin sections of only 2.3 per cent. The mean colorimeter reading was 1.8 per milligram of the original weight of the exposed dentin, and the colorimeter reading was 8.4 per milligram of weight loss.

Finally, nineteen strains of proteolytic bacteria, growing in Douglas broth, were allowed to act separately upon weighed sections of intact dentin under conditions similar to the described experiments. The proteolytic bacteria maintained a mean pH of 7.44, and the exposed dentin sections had an average weight loss of 5.2 per cent. Their mean colorimeter reading was 2.2 per milligram of original weight, and the colorimeter reading was 49.2 per milligram of weight lost during exposure. Previously, the mean colorimeter value of intact dentin had been found to be less than 1.0 per milligram (Burnett and Scherp[27]).

Our attention was turned next toward determining what effects the various types of microorganisms, used in the previous experiments, might have upon the organic matrix of dentin already rendered accessible as indicated by diazotization after decalcification in Morse's solution.[32] After inoculating tubes of suitable broth with the various strains of microorganisms, a section of decalcified dentin was added aseptically to each tube. These cultures were incubated at 37° C., and the condition of the sections of dentin was noted at twenty-four hour intervals, for a total exposure of 120 hours. Decalcified, diazotized sections of dentin, used as controls, were placed in sterile broth adjusted to the average pH maintained by the various strains of bacteria during the previous experiments. The results of these experiments

TABLE II

THE ACTION OF MICROORGANISMS UPON DIAZOTIZED, DECALCIFIED DENTIN

SPECIES	NUMBER OF STRAINS	24 HOURS	48 HOURS	96 HOURS	120 HOURS	CONTROLS 120 HOURS
Lactobacilli	10	10 N	10 N	7 N	10 P	All P
				3 P		
Actinomycetes	10	10 N	10 N	6 N	1 N	All N
				2 P	4 P	
				2 C	5 C	
Cocci	8	8 N	8 N	8 N	8 N	All N
Proteolytic bacteria	19	19 N	11 N	5 N	11 C	All N
			1 P	1 P	8 D	
			5 C	7 C		
			2 D	6 D		

Bacterial strains cultured in the same media as in Table I. One section of dentin per strain of bacteria.

Key: N, No action; P, color partially removed; C, color completely removed; D, disintegrated.

are shown in Table II. In the case of the lactobacilli, ten strains growing in 2 per cent glucose broth were tested, and no change in the dentin was observed for the first forty-eight hours. At the end of ninety-six hours, seven sections were unchanged, while the color in three sections had diminished in intensity but had not been removed from the organic matrix. The color intensity of all the diazotized sections, exposed to lactobacilli, had diminished at the end of 120 hours. The color of control dentinal sections, maintained in broth at pH 3.5, also diminished in intensity. It was found also that the color intensity of other diazotized proteins diminished in acid solutions (pH 3 to 4), but the colored protein substance was not necessarily removed from the bulk of the proteins by such treatment.

To observe the action of the actinomycetes, diazotized, decalcified dentin sections were exposed separately to ten strains growing in thioglycolate broth. All dentin sections were unchanged during the first forty-eight hours. At the end of ninety-six hours, six sections were unchanged, in two sections the color was partially removed, and in two sections the color was completely removed. At the end of 120 hours, one section was unchanged, in four sections the color was partially removed, and in five sections the color was completely removed, but no section was disintegrated. Control sections of dentin, maintained in thioglycolate broth at pH 5.0, were unchanged during the 120-hour test period.

Diazotized, decalcified dentin sections were exposed separately to each of eight strains of cocci in 2 per cent glucose broth. All dentin sections were unchanged after 120 hours. Control sections of dentin were unchanged when maintained in 2 per cent glucose broth at pH 5.6 for 120 hours.

Nineteen strains of oral proteolytic bacteria, growing in Douglas broth, were allowed to act separately on sections of diazotized, decalcified dentin. No change was produced in these sections during the first twenty-four hours. At the end of forty-eight hours' exposure, eleven sections were unchanged, in one section the color was partially removed, in five sections the color was removed, and two sections were disintegrated. At the end of ninety-six hours, five sections were unchanged, in one section the color was partially removed, in seven sections the color was removed, and six sections were disintegrated. At the end of 120 hours, in eleven sections the color was removed and eight sections were disintegrated. All of the control sections of dentin, maintained in Douglas broth at pH 7.5, were unchanged during 120 hours of exposure.

Further experiments were directed toward finding out what effect two proteolytic enzymes, pepsin and trypsin, had upon sections of intact dentin. Pepsin (Merck, U.S.P.) in 0.5 per cent concentration was utilized in several acid-buffer solutions, all of which were adjusted to pH 2.0. The results of the exposure of intact dentin to these solutions are summarized in Table III. In the first experiment, five weighed sections of intact dentin were added separately to 5 ml. portions of the pepsin-hydrochloric acid-potassium chloride solution and maintained at 37° C. for forty-eight hours. The mean weight loss of these sections was 10.5 per cent and the average colorimeter reading was

TABLE III

A SUMMARY OF THE ACTION OF VARIOUS ENZYME-ACID MIXTURES ON DENTIN

ENZYME MIXTURE	pH	PER CENT WEIGHT LOSS	COLORIMETER READING/MG. ORIGINAL WT.	COLOR/MG. WEIGHT LOSS
Pepsin-hydrochloric acid-potassium chloride	2.0	10.5	1.6	20.2
Pepsin-formic acid-sodium citrate	2.0	78.8	10.8	15.2
Pepsin-lactic acid-sodium lactate	2.0	73.0	6.3	9.7
Trypsin	8.0	2.7	1.2	48.7

Five sections of dentin exposed separately to each enzyme solution for forty-eight hours at 37° C.

1.6 per milligram of original weight. The average colorimeter reading was 20.2 per milligram of weight loss during exposure to the enzyme.

In the second experiment, the procedure just outlined was followed except that a pepsin-formic acid-sodium citrate solution was substituted for the pepsin-hydrochloric acid-potassium chloride mixture. In this case, the mean weight loss of the dentin sections was 78.8 per cent and the average colorimeter reading of original weight was 10.8. The colorimeter reading was 15.2 per milligram of weight loss.

In the third experiment, five dentin sections were exposed to a pepsin-lactic acid-sodium lactate mixture under conditions similar to those of the first experiment. These dentin sections had an average weight loss of 73 per cent, a mean colorimeter reading of 6.3 per milligram of original weight, and a mean colorimeter reading of 9.7 per milligram of weight loss.

In the fourth experiment, five weighed sections of intact dentin were exposed separately, for forty-eight hours at 37° C., to 5 ml. of a 0.5 per cent trypsin solution (Pfanstiehl), pH 8.0. The average weight loss of these sections was 2.7 per cent. The average colorimeter reading was 1.2 per milligram of original weight and the mean colorimeter reading was 48.7 per milligram of weight loss.

In a manner similar to these experiments, decalcified, diazotized dentin and naturally carious, diazotized dentin were exposed to several of the 0.5 per cent pepsin and trypsin solutions. In each test five sections of decalcified, diazotized dentin and two sections of diazotized, carious dentin were exposed separately to 5 ml. of the enzyme mixtures for ninety-six hours at 37° C. The

TABLE IV

THE ACTION OF PEPSIN AND TRYPSIN ON SECTIONS OF DIAZOTIZED, DECALCIFIED DENTIN AS COMPARED TO DIAZOTIZED, NATURALLY CARIOUS DENTIN

ENZYME MIXTURE	pH	NO. OF SECTIONS	24 HOURS	48 HOURS	96 HOURS	NATURALLY CARIOUS DENTIN (96 HOURS)
Pepsin-hydrochloric acid-potassium chloride	2.0	5	5 CR	3 CR 2 PD	3 PD 2 D	5 N
Trypsin	8.0	5	5 N	1 N 4 CP	3 CR 2 PD	5 N

Five sections of diazotized, decalcified dentin and two sections of naturally carious dentin were exposed separately to each enzyme solution at 37° C.

Key: N, No action; CR, color removed; PD, partially disintegrated; D, disintegrated.

results of these experiments are shown in Table IV. All of the diazotized dentin sections exposed to a pepsin-hydrochloric acid-potassium chloride mixture had the color removed within twenty-four hours. Whether the diminution in color at this time was due to the strong acidity of the solution or to the action of enzymes is not known. However, at the end of forty-eight hours, two of the dentin sections were partially disintegrated, and at the end of ninety-six hours, two sections were completely disintegrated and three were partially disintegrated. The diazotized, naturally carious dentin was not affected during ninety-six hours' exposure, except for some diminution of color, presumably due to the high acidity.

Five sections of diazotized, decalcified dentin exposed separately to 5 ml. of trypsin were not changed during the first twenty-four hours. At the end of forty-eight hours, one section was unchanged, while the color of four was partially removed. At the end of ninety-six hours, in three sections the color was removed, while two were partially disintegrated. Two sections of diazotized, naturally carious dentin were essentially unchanged during the ninety-six hour test period.

DISCUSSION

While it may be that the carious process in dentin is initiated by the action of one bacterial species, it does not seem likely that such a single bacterial species acts alone in all stages of the destruction of dentin. Various kinds of bacteria have been found in the vicinity of the advancing front in deep dentinal caries. These bacteria possess varying capacities for the production of decalcifying acids and lytic enzymes that could, at the appropriate time, attack, respectively, the inorganic and organic matrix of dentin (Burnett and Scherp[31]). In the present investigation, we have studied the effects in vitro of various types of oral bacteria upon several types of dentin in order to obtain information concerning the action of these bacteria upon both the inorganic and organic portions of this tissue. In a previous study (Burnett and Scherp[27]), it had been noted that the organic matrix of dentin was not accessible for chemical reactivity and lysis until partial decalcification had been accomplished by various organic or inorganic acids. In order to determine whether the same conditions held true in dentin exposed to bacteria, it was necessary to study the effects of oral acidogenic and proteolytic microorganisms upon both the organic and inorganic portions of dentin. When dentin was exposed to the microorganisms in vitro, it was found that the lactobacilli produced the greatest amount of acid and consequently were the most efficient decalcifiers, causing 74 per cent weight loss in twenty-one days. The actinomycetes were the next best acid producers but caused only 15 per cent weight loss during the same period of time. The cocci produced less acid than either the lactobacilli or the actinomycetes and caused only a 2 per cent weight loss of the dentin sections. While the proteolytic bacteria were not acidogenic and presumably were not able to cause decalcification, they did cause a weight loss of slightly over 5 per cent; almost twice that of the cocci.

The organic matrix of dentin, exposed to the various kinds of oral bacteria, was rendered partially accessible for the formation of azoproteins, i.e., rendered accessible for the action of lytic enzymes. The lactobacilli rendered the greatest number of reactive groups accessible, followed in decreasing activity by the actinomycetes, proteolytic bacteria, and cocci. However, the amount of the organic matrix made accessible per unit of original weight of the intact dentin, after twenty-one days' exposure to the test organisms, was in no case as great as that which had been found accessible following exposure for twenty-four hours to acid solutions at pH 3.0. The amount of protein rendered accessible per unit of weight lost during exposure to the microorganisms was quite similar for the lactobacilli and filamentous microorganisms, but was much lower for the cocci, and was much higher for the proteolytic bacteria.

The results of the exposure of decalcified, diazotized dentin (dentin in which the organic matrix had been rendered accessible to proteolytic enzymes) to various oral bacteria in vitro indicate that these bacteria have a variable action on this portion of the tissue. Lactobacilli apparently have a slight action on the diazotized dentin only by the amount and type of acid they produce. If the cocci act on the accessible organic matrix of dentin, they evidently do so rather slowly, analogously to the cocci (mostly streptococci) that produce slow changes in milk protein in cheese making, by either the production of lytic enzymes or organic acids. The actinomycetes acted upon the diazotized dentin to remove the colored protein, but they did not have a demonstrable lytic action. In contrast, most of the proteolytic bacteria were able to lyse this type of dentin quite rapidly.

From the foregoing discussion it is apparent that microorganisms act upon dentinal tissues in several ways. One of the actions of microorganisms on dentinal tissue is decalcification. Decalcification of dentin by the acids produced by oral microorganisms removes its inorganic portion and simultaneously renders its protein matrix accessible to lytic enzymes of several kinds of microorganisms. The removal of the inorganic portions of dentin is apparently essential to the rendering of any significant amount of its organic matrix accessible to the lytic enzymes of the oral bacteria.

Different kinds of oral bacteria render the protein matrix of dentin accessible at different rates. The number of reactive chemical groups rendered accessible in relation to the amount of decalcification is relatively constant for each kind of bacteria, but this amount differs between different kinds of bacteria. Fewer reactive groups are made accessible by the cocci and proteolytic bacteria when compared to those made accessible by the lactobacilli and actinomycetes.

Once the reactive group of the organic matrix of dentin is made accessible, the lytic bacteria of the carious lesion can act upon it. However, the proteolytic bacteria of the carious lesion have some action on the protein matrix of intact dentin without the concurrent action of acids, for the amount of accessible protein of dentin increases upon exposure to these bacteria. They are not able to lyse such dentin. However, in most cases the proteolytic bac-

teria are able to lyse the accessible organic matrix of dentin quite rapidly. Those microorganisms that do not produce a rapidly lytic enzyme may act slightly upon accessible dentinal protein either by the acids they produce or by slower acting lytic enzymes.

The exposure of intact dentin to two typical proteolytic enzymes, pepsin and trypsin, gave variable results. The pepsin solutions were adjusted to pH 2.0 with several of the organic and inorganic acids used for decalcification. As had been noted before (Burnett and Scherp[27]), some acids render the protein matrix accessible at different rates, but the amount of the protein rendered accessible in proportion to the amount of decalcification was found to be relatively constant. When dentin was exposed to acids plus pepsin, decalcification occurred, but the amount of accessible protein per milligram of weight lost varied from one acid-pepsin solution to another. This phenomenon had not been observed when the acids had been used alone, probably because the pepsin digested some of the protein matrix.

Trypsin, acting upon dentin at pH 8.0, resulted in the loss of only a small amount of weight. The colorimeter reading per unit weight loss caused by trypsin was quite high, but this was caused, in part, by the small loss of weight.

Can the organic matrix of decalcified dentin or naturally carious dentin be attacked or lysed by pepsin or trypsin? Dentin decalcified for twenty-one days in Morse's solution was lysed by pepsin or trypsin within a relatively short time. Diazotized, naturally carious dentin was not readily lysed, within the same period of time, by the two proteolytic enzymes. This indicates that dentin decalcified by a relatively strong acid is less resistant to lysis than naturally carious dentin.

TABLE V

A SUMMARY OF THE RATE OF DIAZOTIZATION OF DENTIN EXPOSED TO BACTERIA, ENZYMES, AND ACIDS

TYPE OF TREATMENT	AMOUNT OF DIAZOTIZATION*
Decalcified by organic acids	18.7
NaF treated and decalcified by organic acids	17.2
Lactobacilli	18.3
Filamentous organisms	23.6
Cocci	8.5
Proteolytic bacteria	49.2
Pepsin-hydrochloric acid	20.2
Pepsin-formic acid	15.2
Pepsin-lactic acid	9.7
Trypsin	48.7

*Colorimeter reading per milligram weight loss.

If the amounts of accessible protein per unit of weight loss are compared (Table V), some similarities can be noted in the action of each kind of bacteria and of each enzyme. The action of the organic acids and the lactobacilli on dentin are similar. The action of trypsin and proteolytic bacteria on dentin are comparable but differ from actions in which acids are involved. The action of the cocci and the pepsin-lactic acid mixture are quite alike, but,

in this case, fewer reactive chemical groups are made accessible per unit of weight loss than was obtained by other methods of treatment.

CONCLUSION

The results show that various types of acidogenic bacteria from dentinal caries are able to decalcify intact dentin, thereby producing changes in its organic matrix which make it accessible for reactivity with diazonium salts and lytic enzymes. The acidogenic bacteria are not able to lyse such an accessible organic matrix. On the other hand, proteolytic bacteria from dentinal caries are able to produce only minimal changes in intact dentin, although they readily digest dentinal protein made accessible by acid decalcification. The proteolytic bacteria do not lyse naturally carious dentin as readily as they lyse acid decalcified dentin. The actinomycetes from dentinal caries are able to produce a type of discoloration in noncarious dentin similar to that seen in the dentin of old or arrested caries. Pepsin-acid mixtures produce variable changes in the organic matrix of dentin that correspond somewhat to the action of the acidogenic microorganisms obtained from dentinal caries. Trypsin produces an action on the organic matrix of dentin similar to that produced by the proteolytic bacteria.

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INFLUENCE OF INGESTION OF SINGLE AMINO ACIDS ON THE LEVEL OF FREE AMINO ACIDS IN HUMAN SALIVA

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THE presence of free amino acids in the saliva of human beings on a normal as well as a low protein diet has been demonstrated.[1, 2] Results of these studies indicated that the variation in concentration of these amino acids is not great.

Several reports appeared in the literature concerning studies of the effect of relatively large doses of single amino acids on the blood amino nitrogen level.[3-9] Hier[10] studied the effect of ingestion of a single amino acid on the level of other individual amino acids in the blood.

We now report the effect of the ingestion of one single amino acid taken by mouth on the concentration of that particular amino acid in the saliva.

EXPERIMENTAL

Samples of saliva of two volunteer subjects were collected at various intervals over a twenty-four hour period and analyzed for a particular free amino acid under study. On the following day, the procedure of collection was repeated with the exception that either a cola drink or ice cream was consumed prior to the collection of the first test sample. The reason for this was to rule out any possible effect of these on the amino acid content of the saliva, since the large doses of the individual amino acid were mixed with either a cola drink or ice cream when these acids were taken internally. It was found that neither vehicle influenced the normal free amino acid values of saliva. On the third day, one sample of saliva was collected prior to the ingestion of the amino acids; 5 Gm. was then taken orally, the mouth rinsed thoroughly with water, which was swallowed, and after a two-minute interval, the first of the test samples of the saliva was collected. Additional samples were collected at various intervals during a twenty-four hour period.

The following free amino acids were determined microbiologically by the methods previously described[1, 2]: isoleucine, tryptophane, valine, threonine, cystine, lysine, arginine, leucine, histidine, tyrosine, phenylalanine, and methionine. The concentrations of each of these, expressed in micrograms per milliliter of saliva, over the periods studied are shown in Table I.

Fig. 1 shows the results obtained on phenylalanine when this amino acid was fed in 5 and 10 Gm. quantities. It further shows that the tyrosine level of saliva did not go up when the phenylalanine was administered. This is in contrast to the observation of Hier[10] when he studied plasma values of amino acids. The graph is typical of the general results obtained when the other amino acids were administered orally in the 5 Gm. quantity.

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TABLE I

AMINO ACID LEVELS IN SALIVA

AMINO ACID	PRIOR TO INTAKE	AFTER INTAKE OF 5 GM. OF THE PARTICULAR AMINO ACID				
		2 MIN.	1 HR.	3 HR.	5 HR.	24 HR.
Cystine	*a–0.2	6.5	2.0	0.3	0.2	0.4
	b–0.4	7.0	1.2	0.4	0.5	0.6
Phenylalanine	1.2	14.5	4.0	1.5	1.0	1.8
	2.4	13.0	2.0	4.5	2.5	3.0
Tyrosine	0.6	25.0	3.5	6.0	2.1	1.4
	0.4	25.5	5.5	7.0	1.8	2.1
Histidine	2.4	83.0	4.5	3.0	2.5	1.5
	1.2	62.0	6.8	2.0	1.5	2.0
Methionine	0.05	7.5	1.0	0.5	0.05	0.05
	0.1	9.5	1.2	0.6	0.3	0.05
Leucine	0.7	145.0	2.0	0.6	0.5	0.4
	0.5	56.0	0.8	1.0	0.4	1.2
Isoleucine	0.2	35.0	0.2	0.5	0.6	1.1
	0.7	25.0	0.3	0.4	0.9	0.8
Tryptophane	0.4	50.0	0.3	0.5	0.8	0.6
	0.8	47.5	1.3	0.4	1.0	0.9
Valine	1.6	79.0	2.0	1.0	1.2	0.9
	2.4	30.0	1.8	2.0	1.5	3.1
Threonine	0.5	18.0	0.5	0.8	0.6	0.5
	2.4	14.0	0.8	1.0	1.2	1.5
Lysine	0.2	60.0	0.7	1.0	0.9	0.5
	1.2	102.0	0.8	1.3	1.1	0.9
Arginine	7.5	300.0	4.5	7.0	3.5	5.5
	10.2	130.0	10.5	12.2	8.0	9.3

Concentrations expressed in micrograms per milliliter of saliva.
*a—subject, E. B.; b—subject, J. J.

DISCUSSION

The saliva levels of all of the free amino acids were found to rise significantly following the ingestion of the corresponding amino acid. The concentrations were back to normal levels within about thirty to sixty minutes. Thorough rinsing of the mouth after the ingestion eliminated any possibility of some of the acids remaining in the oral cavity after the consumption. In several cases repeated rinsing over a five-minute period using several fresh portions of water as well as thorough brushing without the use of a dentifrice did not produce any results different from those obtained following the two-minute rinsing period.

For reasons of tolerance, the 5 Gm. portions of the acids were used in all of the experiments after the subjects began to complain about nausea when 10 Gm. portions were ingested.

In these cases where both 5 and 10 Gm. quantities of the amino acids were used (cystine, tyrosine, and phenylalanine), the peak values were higher with the 10 Gm. portions; however, after one hour, all of the levels were down to normal again.

Our results on saliva are of interest in view of the findings of Hier on plasms.[10] He reported the plasma values in general to be higher than normal for a period of at least five hours after ingestion, while the peak was reached after about two to three hours. In a number of cases, even after twenty-four hours, the plasma levels of amino acids were higher than normal. In the case

of cystine, Hier did not find any appreciable increase. A correlation between normal values of human saliva as compared with those reported by Hier and Bergeim[11] of plasma could not be established. The normal plasms levels are much higher.

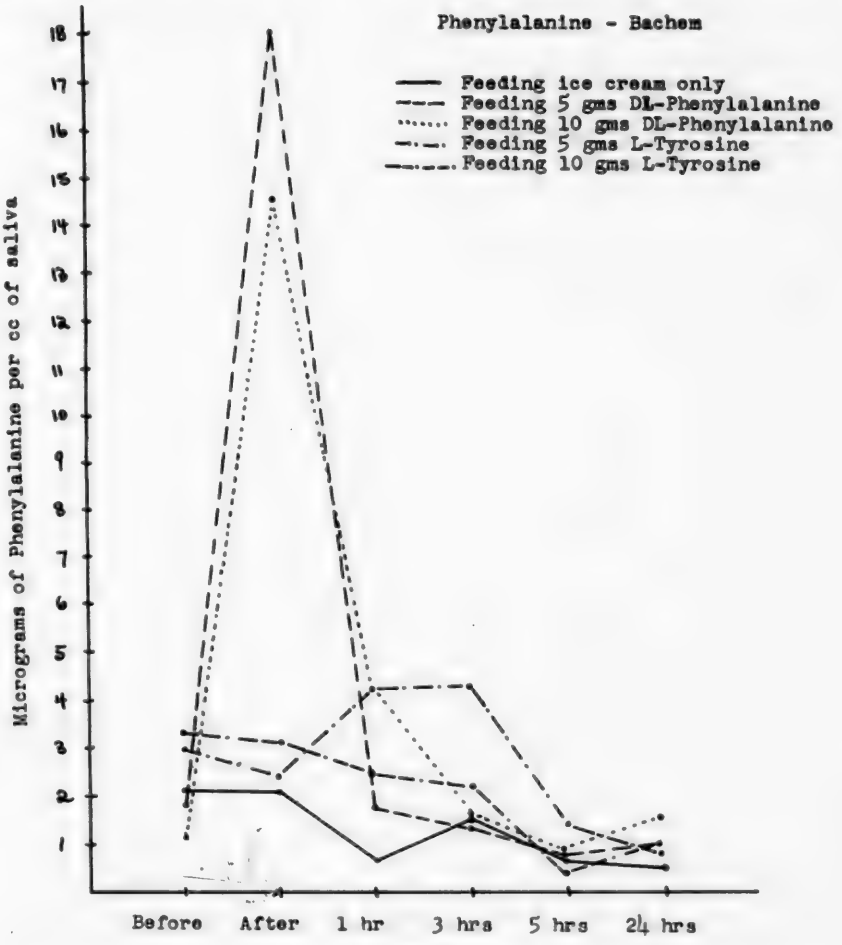

Fig. 1.

Hier[10] also found the tyrosine level to rise in the plasma when phenylalanine was ingested, while the reverse did not occur. This rapid, well-recognized conversion of phenylalanine to tyrosine, while it shows in the plasma as studied by Hier,[10] in our saliva studies was not observed.

The ingestion of methionine, which presumably is a source of cystine in the body, did not cause an increase in the plasma level of cystine.[10] We did not find any increase in cystine in saliva when methionine was fed, while level of the latter followed the general pattern for all of the amino acids tested.

SUMMARY

The saliva level of isoleucine, tryptophane, valine, threonine, cystine, lysine, arginine, leucine, histidine, tyrosine, phenylalanine, and methionine rose when the corresponding amino acid was given orally. After thirty to sixty minutes, the values returned to normal. Conversion of phenylalanine to tyrosine was not observed as reflected in saliva values.

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EFFECT OF THREE DIPHENYLMETHANE DERIVATIVES ON ACID PRODUCTION IN SALIVA AND ON LOGARITHMIC GROWTH OF AN ORAL LACTOBACILLUS

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DIHYDROXYHEXACHLORODIPHENYLMETHANE (G-11) has been shown by Traub, Newhall, and Fuller[3] and by Seastone[2] to reduce markedly the total flora of the skin for prolonged periods. The duration of the activity was ascribed by Fahlberg, Swan, and Seastone[1] to retention of the chemical in or on the tissue.

This investigation was undertaken to determine if G-11 and two related compounds, dihydroxyquadrochlorodiphenylmethane (XDR-504) and dihydroxydichlorodibromodiphenylmethane (XDR-505), could alter the logarithmic growth of an oral lactobacillus or influence acid production in pooled saliva.

MATERIALS

Each agent was prepared in the following manner. One-tenth gram of the C. P. solid was dissolved in approximately 15 ml. of 95 per cent ethanol with gentle heating. The solution was transferred quantitatively to a volumetric flask and ethanol added to a final volume of 100 ml. Upon cooling, the volume was adjusted and approximately 60 ml. transferred to a vaccine vial which was immediately sealed with an airtight rubber stopper. One milliliter syringes, graduated in hundredths, provided with 20 gauge needles, were used for dispensing. Thus, while delivering quantities of reasonable accuracy, alteration in concentration by evaporation of ethanol was precluded.

The oral lactobacillus employed was isolated on Hadley's tomato juice agar and maintained in acid glucose broth at 37° C. with transfers at twenty-four hour intervals. Under such conditions, the lag phase of the culture approximated four hours. All pour plates were prepared with Snyder's B.C.G. Dextrose Agar (Difco Laboratories, Incorporated).

Pooled saliva was obtained from workers in the laboratory. It was collected by paraffin stimulation, immediately frozen, and used within eighteen hours.

METHODS

Influence of Agents on Logarithmic Growth.—Following several acid glucose broth transfers at twenty-four hour intervals, 0.1 ml. of the culture was seeded into 30 ml. of broth. After five hours' incubation, sufficient interval to allow for the four-hour lag phase, 1 ml. of the culture was inoculated into a series of glucose broth tubes, each of which contained one of the agents under test. The extremes of concentration were 0.125 and 0.5 μ per milliliter. Six

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control tubes were also seeded, one containing the volume of ethanol used with the highest concentration of test agent, one containing the volume used with the lowest concentration, and four unaltered. From this last group, duplicate pour plates at $1:1 \times 10^4$ were made from each of two tubes to estimate the number of lactobacilli at time zero. All remaining tubes were incubated for

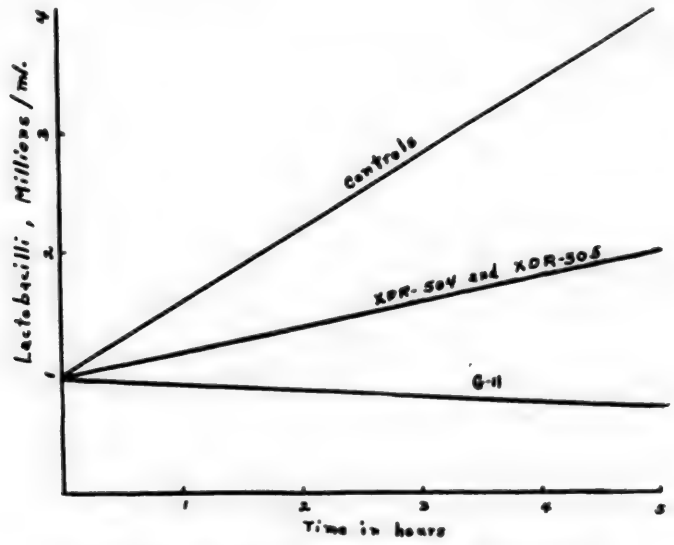

Fig. 1.—Effect of test agents at 0.125 μ per milliliter.

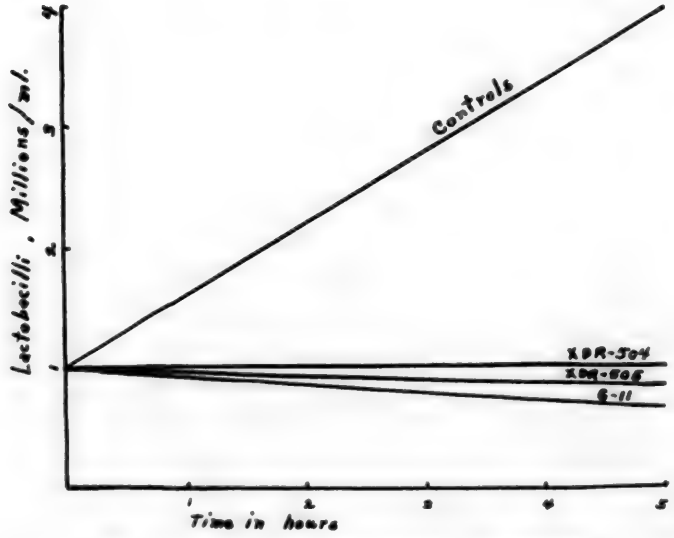

Fig. 2.—Effects of test agents at 0.5 μ per milliliter.

five hours, at which time duplicate pour plates at $1:1 \times 10^6$ were prepared from each tube.

Influence of Test Agents on Acidogenesis in Pooled Saliva.—Pooled saliva, disregarding alteration due to carbon dioxide loss, exhibited a reaction between pH 7.0 to pH 7.5. Marked alkalinity occurred following short incubation at 37° C. However, if mixed 1:1 with an aqueous solution containing dextrose 1 per cent and peptone 0.5 per cent, acid production was evident within two hours and continued for periods up to six hours. Each of the three chemicals was added to separate 5 ml. portions of the dextrose-peptone solution, autoclaved at 121° C. for fifteen minutes, cooled, and to each portion 5 ml. of pooled saliva added. The final concentrations of the test substances ranged from 1 to 20 μ per milliliter. Initial reaction was determined for each sample and the preparations were incubated for five hours, during which time pH determinations were made at varying intervals.

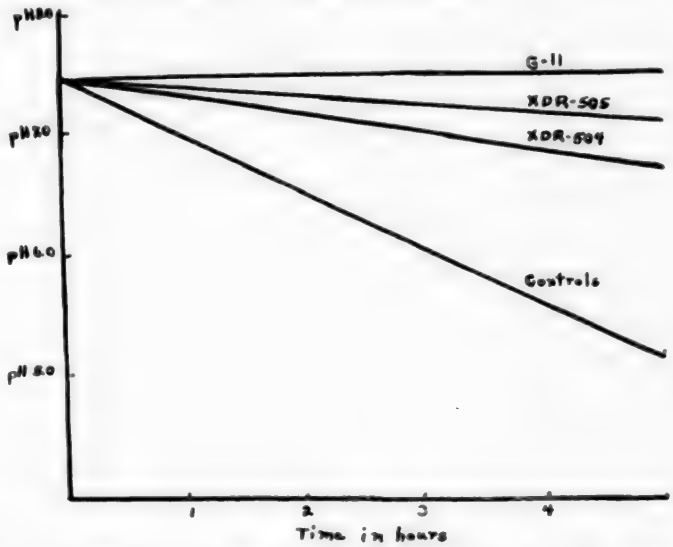

Fig. 3.—Acidogenesis in saliva altered by test agents at 20 μ per milliliter.

The actions of the diphenylmethane derivatives upon the logarithmic growth of the lactobacillus and upon acid production in pooled saliva dextrose-peptone were considered on the average of three trials with each agent at a given concentration, all other factors constant.

RESULTS

Fig. 1 illustrates the effect of the agents at a concentration of 0.125 μ per milliliter upon the logarithmic growth of the oral lactobacillus. Fig. 2 shows the results at 0.5 μ per milliliter.

Fig. 3 indicates the extent to which acidogenesis in pooled saliva dextrose-peptone was inhibited by the compounds at a concentration of 20 μ per milliliter. At lower concentrations the inhibition was much less effective.

CONCLUSIONS

Dihydroxyquadrochlorodiphenylmethane (XDR-504), dihydroxydichlorodibromodiphenylmethane (XDR-505), and dihydroxyhexachlorodiphenylmethane (G-11), in high dilutions, are capable of altering the logarithmic growth of an oral lactobacillus and are able to inhibit acid production in saliva dextrose-peptone mixtures. At a given concentration the greatest effect is produced by G-11.

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OBSERVATIONS ON THE RELATIONSHIP BETWEEN SELECTED B VITAMINS AND ACID PRODUCTION BY MICROORGANISMS ASSOCIATED WITH HUMAN DENTAL CARIES

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MANN, Dreizen, Spies, and Hunt,[1] in 1947, found that persons with multiple B vitamin deficiencies of many years standing had little or no dental caries activity. Subsequently, Dreizen, Reed, and Spies[2] noted that acid production by oral flora in saliva-glucose mixtures is related to the nicotinic acid content of this secretion. Human saliva contains, in addition to nicotinic acid, variable amounts of thiamine, riboflavin, pyridoxine, pantothenic acid, folic acid, and biotin.[3, 4] Because of their growth-promoting properties and coenzyme functions, differences in the availability and usability of these vitamins may constitute a prime limiting factor in the acid-producing microbial spectrum of the dentobacterial plaque. Acidogenic oral lactobacilli, streptococci, staphylococci, and yeasts have been singled out as possible initiators of or contributors to the caries process.[5-13] Microorganisms differ in their B vitamin requirements and in their ability to synthesize these compounds or to utilize closely related chemical substances. The extent to which the oral acidogens differ in this respect has been determined by a series of investigations designed to establish: (a) the B vitamin needs of representative species of oral acid-producing microorganisms; (b) the effect of the deletion of each of the B vitamins present in human saliva on acid production by oral acidogenic flora; (c) the effect of the addition of a metabolic analogue of nicotinic acid on the growth of pure and mixed cultures of selected oral acidogens; and (d) the effect of the addition of this agent on acid production in human saliva.

EXPERIMENT I

The B Vitamin Requirements of Oral Acidogenic Microorganisms

Method.—Pure cultures of *Lactobacillus acidophilus, Streptococcus salivarius, Staphylococcus albus,* and a glucose-fermenting strain of saccharomyces served as test organisms for the elucidation of the B vitamin requirements of representative species of the various groups of oral acidogens. Each of these organisms was isolated from human saliva and identified by conventional bacteriologic methods. The synthetic chemically defined medium described by Landy and Dicken[14] comprised the test broth (Table I). It was modified for this study by the addition of 4 μg of inositol per 10 c.c. of broth in the tubes inoculated with the oral strain of glucose-fermenting saccharomyces, since exogenous inositol is a growth factor for many strains of saccharomyces.[15]

The specific B vitamin needs of each of the test organisms was established by comparing the amount of acid produced in tubes containing 10 c.c. of com-

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plete test medium with that formed in tubes containing the same amount of test broth devoid of either biotin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, riboflavin, or thiamine. One drop of a physiologic saline suspension prepared from a 24-hour yeast extract-dextrose broth culture comprised the inoculum for each organism. The method of preparation of these inocula was identical with that previously described in detail.[16] Determinations of acid production were made in 5 series of tubes. Each series contained 6 tubes of complete medium and 6 tubes of each of 7 broths of identical composition from which a single B vitamin was withheld. Four series were inoculated with a pure culture of one of the test organisms, the fifth, with a mixed culture of all 4 test organisms. All tubes were incubated at 37° C. Acid production was measured at the end of 24, 48, and 72 hours in terms of the amount of 0.1N NaOH required to raise the pH of the inoculated tubes to that of uninoculated control tubes. A Beckman pH meter was used for this purpose.

TABLE I

COMPOSITION OF TEST MEDIUM*

CONSTITUENT	QUANTITY
Casein hydrolysate	2.5 ml.
Salt solution A†	0.5 ml.
Salt solution B‡	0.5 ml.
Glucose	1. Gm.
Sodium acetate	600 mg.
Asparagine	25 mg.
Tryptophane	10 mg.
Cystine	10 mg.
Guanine hydrochloride	500 μg.
Adenine sulfate	500 μg.
Xanthine	500 μg.
Uracil	500 μg.
Pyridoxine hydrochloride	40 μg.
Riboflavin	20 μg.
Calcium pantothenate	20 μg.
Nicotinic acid	20 μg.
Thiamine hydrochloride	10 μg.
Folic acid	1 μg.
Biotin	0.5 μg.
Distilled water to 50 ml.	

*Double strength, Landy and Dicken medium adjusted to pH 6.8 with NaOH.

†Salt solution A:

K_2HPO_4	5 Gm.
KH_2PO_4	5 Gm.
Distilled water to 50 ml.	

‡Salt solution B:

$MgSO_4 \cdot 7H_2O$	10 Gm.
NaCl	0.5 Gm.
$FeSO \cdot 7H_2O$	0.5 Gm.
$MnSO_4 \cdot 2H_2O$	0.337 Gm.
Distilled water to 250 ml.	

Observations.—The effect of the deletion from the test medium of each of the 7 B vitamins present in saliva on acid production by the test organisms is shown in Fig. 1. Acid formation by the oral strain of *Lactobacillus acidophilus* at the end of 72 hours was inhibited from 96 to 100 per cent when either nicotinic acid, pantothenic acid, thiamine, or biotin was withheld from the complete medium. Each of the 7 B vitamins was required by the oral strain of *Streptococcus salivarius* for maximum acid production; the deletion of either

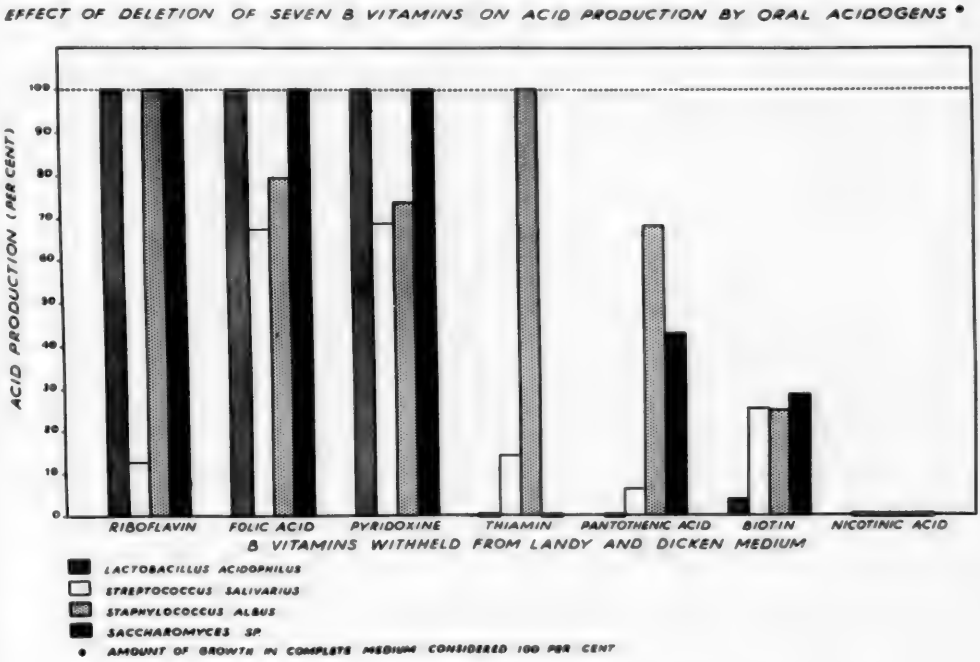
EFFECT OF DELETION OF SEVEN B VITAMINS ON ACID PRODUCTION BY ORAL ACIDOGENS *
ACID PRODUCTION (PER CENT)
100
90
80
70
60
50
40
30
20
10
0
RIBOFLAVIN
FOLIC ACID
PYRIDOXINE
THIAMIN
PANTOTHENIC ACID
BIOTIN
NICOTINIC ACID
B VITAMINS WITHHELD FROM LANDY AND DICKEN MEDIUM
LACTOBACILLUS ACIDOPHILUS
STREPTOCOCCUS SALIVARIUS
STAPHYLOCOCCUS ALBUS
SACCHAROMYCES SP.
* AMOUNT OF GROWTH IN COMPLETE MEDIUM CONSIDERED 100 PER CENT

Fig. 1.

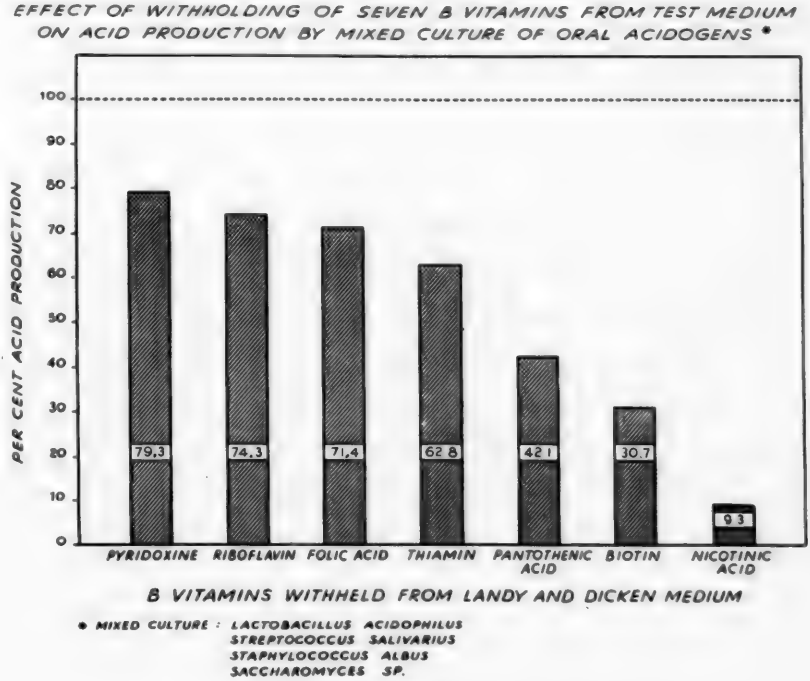
EFFECT OF WITHHOLDING OF SEVEN B VITAMINS FROM TEST MEDIUM
ON ACID PRODUCTION BY MIXED CULTURE OF ORAL ACIDOGENS *
PER CENT ACID PRODUCTION
100
90
80
70
60
50
40
30
20
10
0
79.3
74.3
71.4
62.8
42.1
30.7
9.3
PYRIDOXINE
RIBOFLAVIN
FOLIC ACID
THIAMIN
PANTOTHENIC ACID
BIOTIN
NICOTINIC ACID
B VITAMINS WITHHELD FROM LANDY AND DICKEN MEDIUM
* MIXED CULTURE : LACTOBACILLUS ACIDOPHILUS
STREPTOCOCCUS SALIVARIUS
STAPHYLOCOCCUS ALBUS
SACCHAROMYCES SP.

Fig. 2.

folic acid or pyridoxine had the least effect. Nicotinic acid and biotin were essential for the growth of the oral strain of *Staphylococcus albus*, whereas, nicotinic acid, biotin, and thiamine were required by the glucose-fermenting strain of saccharomyces. Significant reductions in acid formation ranging from 58 per cent to 91 per cent were observed when either nicotinic acid, biotin, or pantothenic acid was withheld from an otherwise complete broth seeded with a mixed culture of all 4 test organisms (Fig. 2).

EXPERIMENT II

The Effect of the Deletion of Various B Vitamins on Acid Production by Mixed Oral Acidogenic Flora

Method.—The effect of the deletion of each of the seven B vitamins present in human saliva on acid production by mixed oral acidogenic flora was determined on 15 samples of paraffin-stimulated saliva obtained from patients with a history and evidence of active dental caries. The generic identity of the acidogenic microorganisms contained in each saliva sample was established by plating 0.1 c.c. of a 1:10 dilution of saliva in physiologic saline on tomato juice agar adjusted to pH 6.1. The mixed flora of each sample was cultured in yeast extract-dextrose broth, inoculated with one drop of whole saliva per 10 c.c. of broth. The tubes were incubated at 37° C. for 24 hours, after which physiologic saline suspensions of the cultures were prepared. These were used to inoculate tubes containing the complete medium and tubes containing this medium from which one of each of the seven B vitamins was withheld. The subsequent treatment of these tubes was identical with that described in Experiment I.

Observations.—Each of the mixtures of oral acidogens obtained from the saliva of 15 caries-active patients required nicotinic acid, pantothenic acid, and biotin for maximal acid production. Six of the 15 cultures required thiamine. None required folic acid, pyridoxine, or riboflavin (Fig. 3).

TABLE II

DISTRIBUTION OF ACIDOGENIC MICROORGANISMS IN INOCULA PREPARED FROM 15 SAMPLES OF HUMAN SALIVA

SAMPLE NUMBER	MICROORGANISM				
	LACTOBACILLUS	STREPTOCOCCUS	STAPHYLOCOCCUS	YEAST	OTHER
1	√	√		√	
2	√			√	Gram-negative bacillus
3	√	√			Gram-negative coccus
4	√		√		
5	√	√			
6	√	√		√	
7	√	√			
8	√		√		Gram-positive diplococcus
9	√	√			
10	√	√			
11	√	√		√	
12	√	√	√		
13	√		√	√	Gram-negative coccus
14	√	√			
15	√		√	√	

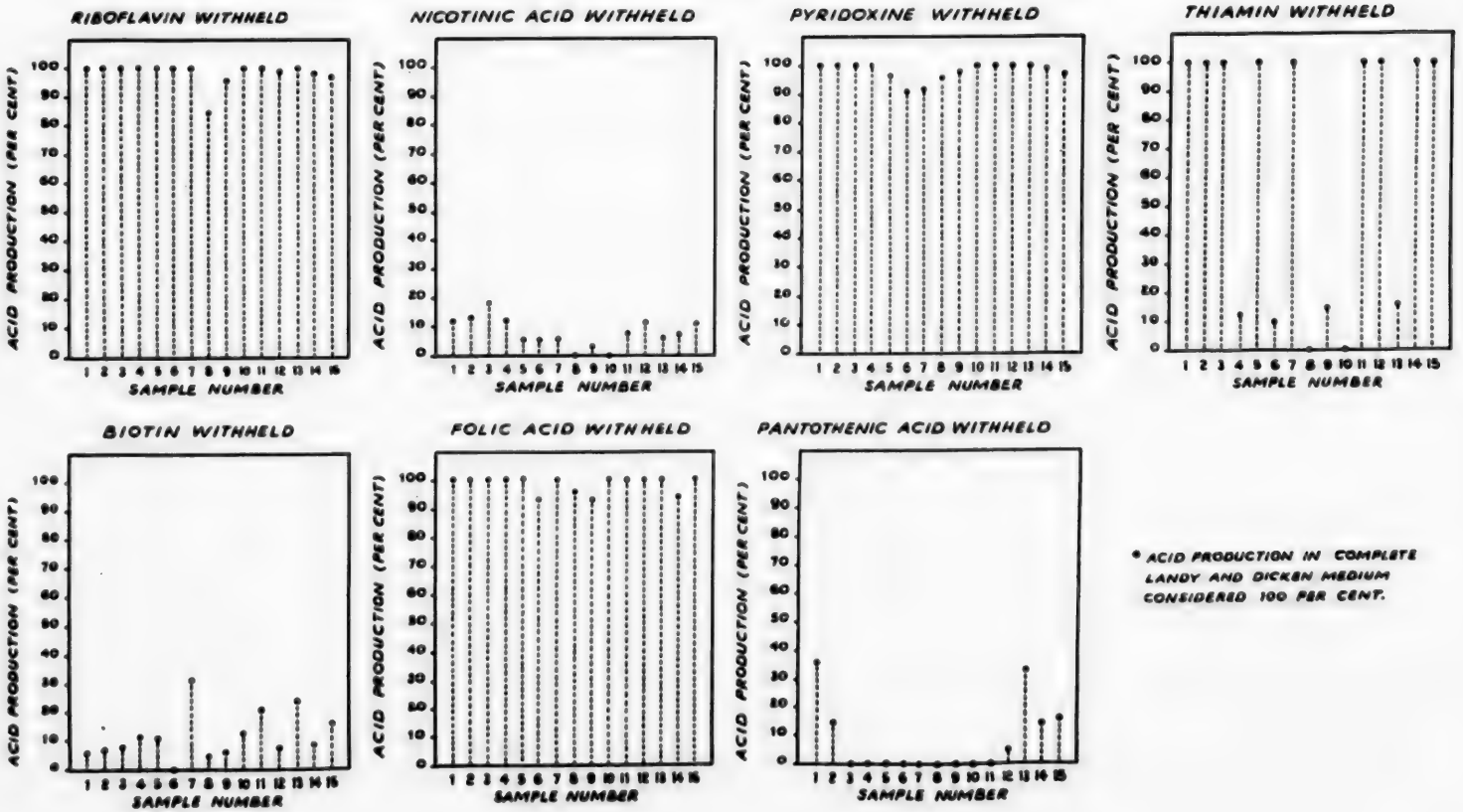
EFFECT OF DELETION OF SEVEN B VITAMINS ON ACID PRODUCTION BY ORAL FLORA *
RIBOFLAVIN WITHHELD
NICOTINIC ACID WITHHELD
PYRIDOXINE WITHHELD
THIAMIN WITHHELD
BIOTIN WITHHELD
FOLIC ACID WITHHELD
PANTOTHENIC ACID WITHHELD
ACID PRODUCTION (PER CENT)
SAMPLE NUMBER
* ACID PRODUCTION IN COMPLETE LANDY AND DICKEN MEDIUM CONSIDERED 100 PER CENT.

Fig. 3.

The distribution of the acidogenic organisms in each of the 15 samples of saliva is shown in Table II. Lactobacilli were present in all 15 samples; streptococci, staphylococci, and yeasts were noted in 10, 5, and 6 samples, respectively. In addition, two samples contained acidophilic gram-negative cocci; one, acidophilic gram-negative bacilli; and one, acidophilic gram-positive diplococci.

EXPERIMENT III

The Effect of a Metabolic Analogue of Nicotinic Acid on Acid Production by Selected Oral Acidogens

Method.—The degree to which the growth of oral strains of *Lactobacillus acidophilus, Streptococcus salivarius, Staphylococcus albus* and glucose-fermenting saccharomyces may be affected by the addition of a metabolic analogue of a member of the B group of vitamins was determined by adding pyridine-3-sulfonic acid in a metabolite: antimetabolite ratio of 1:2500 to tubes of complete test medium seeded with pure and mixed cultures of these organisms. Pyridine-3-sulfonic acid is a metabolic competitor of nicotinic acid for some species of bacteria. The effect of this agent was measured in terms of the difference in amount of acid produced in the tubes containing pyridine-3-sulfonic acid and in those devoid of this compound.

Observations.—The effect of the addition of 10 mg. of pyridine-3-sulfonic acid to broth containing all of the presently known nutritional essentials of the oral strains of the 4 test organisms is illustrated in Fig. 4. The quantities of acid formed by the *Streptococcus salivarius, Staphylococcus albus,* and glucose-fermenting strain of saccharomyces in unmodified complete test broth at the end of 72 hours equaled 54 per cent, 57 per cent, and 18 per cent, respectively, of that produced by the *Lactobacillus acidophilus* under the same experimental conditions. The acid formed in the tubes inoculated with a mixed culture of all four organisms was equal in amount to 95 per cent of that produced by the pure culture of *Lactobacillus acidophilus.* In the amount used, pyridine-3-sulfonic acid completely inhibited the growth of the test strain of *Streptococcus salivarius.* Acid production in the tubes containing pyridine-3-sulfonic acid, which were inoculated with the test strains of *Lactobacillus acidophilus, Staphylococcus albus,* and a mixture of the 4 test organisms, was reduced 33 per cent, 68 per cent, and 62 per cent, respectively. No effect was observed in the tubes seeded with the strain of glucose-fermenting saccharomyces.

EXPERMENT IV

The Effect of the Addition of a Metabolic Analogue of Nicotinic Acid on Acid Production in Human Saliva

Method.—To determine whether pyridine-3-sulfonic acid exerts an inhibitory action on acid production in saliva, 10 mg. of this agent was added to 10 c.c. aliquots from samples of paraffin-stimulated saliva obtained from 25 patients with a moderate to high degree of dental caries activity. The treated aliquots and untreated 10 c.c. aliquots of the same saliva samples were then tested for acid production by the method of Fosdick, Hansen, and Epple.[17]

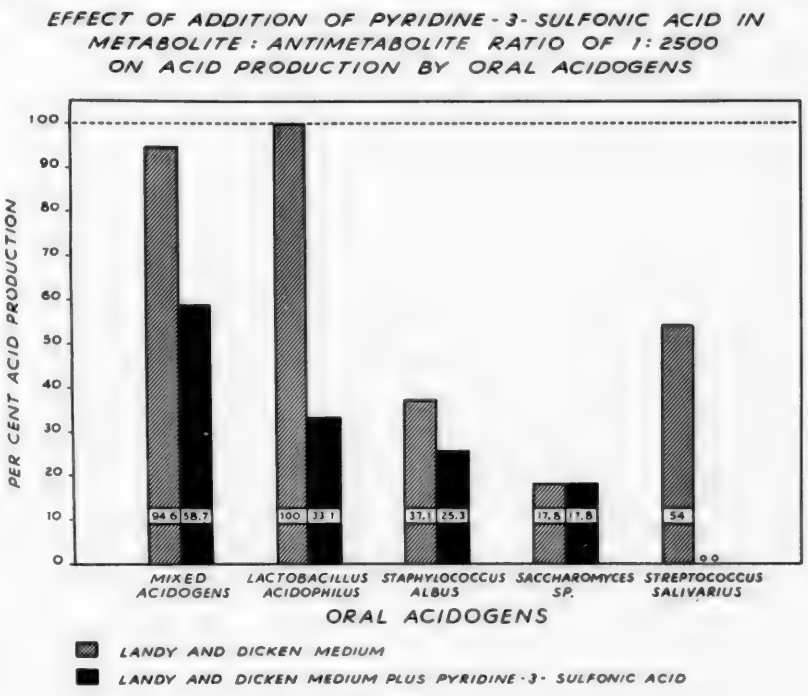
EFFECT OF ADDITION OF PYRIDINE-3-SULFONIC ACID IN METABOLITE : ANTIMETABOLITE RATIO OF 1:2500 ON ACID PRODUCTION BY ORAL ACIDOGENS
PER CENT ACID PRODUCTION
100
90
80
70
60
50
40
30
20
10
0
94.6
58.7
100
33.1
37.1
25.3
17.8
17.8
54
0.0
MIXED ACIDOGENS
LACTOBACILLUS ACIDOPHILUS
STAPHYLOCOCCUS ALBUS
SACCHAROMYCES SP.
STREPTOCOCCUS SALIVARIUS
ORAL ACIDOGENS
LANDY AND DICKEN MEDIUM
LANDY AND DICKEN MEDIUM PLUS PYRIDINE-3-SULFONIC ACID

Fig. 4.

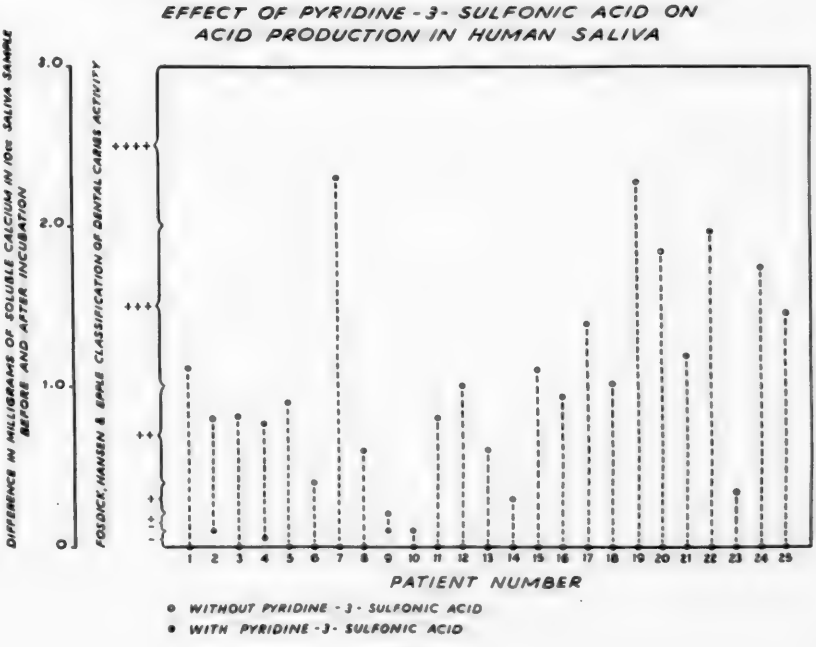
EFFECT OF PYRIDINE-3-SULFONIC ACID ON ACID PRODUCTION IN HUMAN SALIVA
DIFFERENCE IN MILLIGRAMS OF SOLUBLE CALCIUM IN 10cc SALIVA SAMPLE BEFORE AND AFTER INCUBATION
FOLDICK, HANSEN & EPPLE CLASSIFICATION OF DENTAL CARIES ACTIVITY
3.0
2.0
1.0
0
++++
+++
++
+
±
-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
PATIENT NUMBER
WITHOUT PYRIDINE-3-SULFONIC ACID
WITH PYRIDINE-3-SULFONIC ACID

Fig. 5.

Observations.—Pyridine-3-sulfonic acid completely inhibited acid production in each of the 25 samples of caries-active saliva. The distribution of the dental caries activity in the untreated aliquots is shown in Fig. 5.

DISCUSSION

The acidogenic microorganisms associated with human dental caries are dependent upon various members of the B group of vitamins for growth and acid production. The different species and different strains of the same species differ in their need for specific B vitamins. Pure cultures of oral strains of *Lactobacillus acidophilus, Streptococcus salivarius, Staphylococcus albus,* and glucose-fermenting saccharomyces, as well as mixed cultures of generically identical organisms obtained from salivas of caries-active patients, all showed a unanimity of need for nicotinic acid, pantothenic acid, and biotin. Thiamine was a growth essential for 6 of the 15 mixed cultures containing lactobacilli. The pure cultures of *Staphylococcus albus* and *Streptococcus salivarius* failed to attain maximal acid production in the absence of thiamine and riboflavin, respectively. Smiley, Niven, and Sherman[18] noted that riboflavin is a growth essential for *Streptococcus salivarius.* It is apparent, therefore, that the absence or deficiency of various B vitamins in saliva is a limiting factor in the acidogenic component of the dental plaque.

The addition of pyridine-3-sulfonic acid in a metabolite: antimetabolite ratio of 1:2500 completely inhibited the growth of the oral strain of *Streptococcus salivarius* and significantly reduced acid formation by pure cultures of *Lactobacillus acidophilus* and *Staphylococcus albus.* This compound had no effect on acid production by a pure culture of an oral strain of saccharomyes grown in a synthetic medium, but completely inhibited acid production in all samples of caries-active saliva containing generically similar organisms. It would thus appear that there are species and strain-specific differences among the oral acidogens in their vulnerability to substances with molecular configurations approximating those of essential nutrients.

SUMMARY AND CONCLUSIONS

1. Several members of the B group of vitamins are indispensable for acid production by the oral flora associated with human dental caries.

2. Acid production by pure and mixed cultures of oral strains of lactobacilli, streptococci, staphylococci, and yeasts was completely or substantially inhibited when either niotinic acid, pantothenic acid, or biotin was deliberately withheld from an otherwise nutritionally adequate synthetic medium. Thiamine and riboflavin were essential growth factors for several strains of these organisms.

3. The oral acidogens showed species-specific differences in their susceptibility to the inhibitory action of pyridine-3-sulfonic acid, a metabolic analogue of nicotinic acid.

4. Species- and strain-specific differences in B vitamin requirements may be a contributing factor to the variations in the acidogenic component of the dental plaque observed in different persons and in the same person at different times. This factor may help to explain the inconsistencies encountered in

attempts to incriminate a single specific microorganism as the cause of dental caries.

We are indebted to Ellie Jo Gilley, Janet Mosny, and Ann Reed for their technical assistance in making these studies.

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EXPERIMENTAL DENTAL CARIES IN THE WHITE-TAILED RAT IN SOUTH AFRICA

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INTRODUCTION

DURING the last thirty years, numerous experiments in connection with dental caries have been conducted in different countries in the world with the white rat, the cotton rat, and the golden hamster. Only the white rat, bred from imported stock, has been used in South Africa, because the cotton rat and the hamster are not indigenous to the country and they are difficult to obtain. For this reason the question of using other South African indigenous rats, in which the coarse, hard particle is not necessary to initiate caries, was investigated. The research officer in charge of the plague research laboratory attached to the Union Health Department suggested that the white-tailed rat, *Mystromys albicaudatus* (Smith), might answer the purpose. A number of these rats were accordingly made available by this laboratory for experimental dental caries research.

Mystromys albicaudatus is a gray, white-tailed rat, about the size of the golden hamster (Fig. 1). It is a nocturnal, solitary, burrowing rodent, which is widely but sparsely distributed throughout the high veld of the Transvaal, Orange Free State, Natal, and along the coastal belt of the Cape in South Africa. It is the only known representative of the subfamily Cricetinae in Africa; its nearest allies appear to be the hamster of Europe and Asia, but it may be more closely related to certain South American cricetines (Ellerman,[2] 1941). It breeds slowly, but steadily in captivity. Females bear their first litters between the ages of 5 and 8 months; the number in each litter averages 3, most of which survive to weaning. Litters are born to mated pairs every five to six weeks; some individual females have produced as many as 27 litters in a lifetime. Unlike the golden hamster, it has no cheek pouches. Its dental formula is the same as that of the white rat, but the molars differ from those of the latter, in that there are no deep grooves running across the occlusal surface buccolingually. The grooves are short and deep and are situated on the buccal and lingual surfaces of the crown (Figs. 2 and 3).

EXPERIMENTAL

Two groups, A and B, of twenty rats each were fed the diets shown in Table I for twenty months.

After 20 months these rats were sacrificed and their molars examined for caries. No caries developed in any of these rats. In order to ascertain if a caries-susceptible strain could be bred, a number of female rats were mated and then fed the high sugar diet of the A group during pregnancy and lacta-

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Fig. 1.—The white-tailed rat.

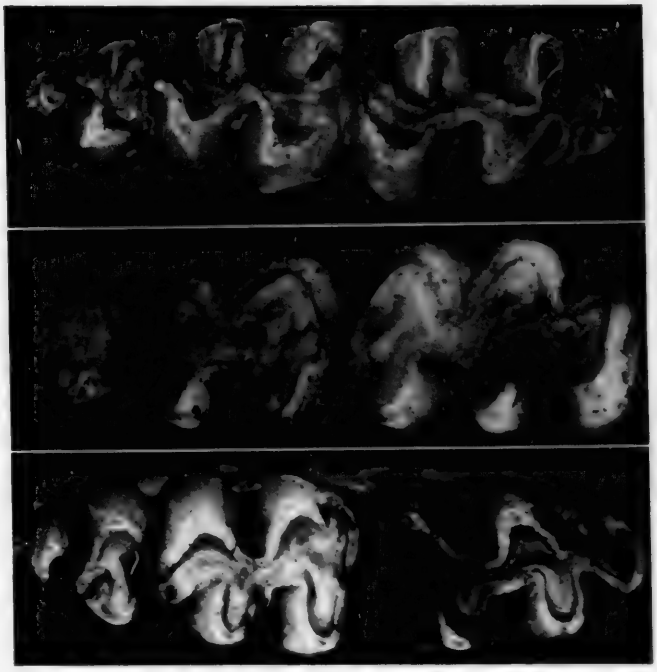

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 2.—Lower molars of the white-tailed rat.
Fig. 3.—Upper molars of the white-tailed rat.
Fig. 4.—Decayed lower molar.

TABLE I

GROUP A		GROUP B	
Sugar	79%	Sugar	40%
Casein	8%	Casein	25%
Food yeast	5%	Food yeast	5%
Salt mixture	4%	Salt mixture	4%
Fish liver oil	4%	Whale meat meal	22%
		Fish liver oil	4%

tion. Two groups, C and D, consisting of seven and eleven rats, respectively, offspring of these females, were fed the diets shown in Table II for eleven months.

TABLE II

GROUP C		GROUP D	
Coarse yellow corn meal	40%	Whale meat meal	10%
Sugar	30%	Sugar	60%
Linseed meal	15%	Linseed meal	15%
Alfalfa meal	2%	Alfalfa meal	2%
Bone meal	2%	Bone meal	2%
Milk powder	5%	Milk powder	5%
Brewers' yeast	3%	Brewers' yeast	3%
Fish liver oil	2%	Fish liver oil	2%
NaCl	.5%	NaCl	.5%
$CaCO_3$	.5%	$CaCO_3$	.5%

Two groups, E and F, consisting of seven rats each, offspring of the second litters of the aforementioned females who were also fed on the high sugar diet during pregnancy and lactation, were fed the same diets of Groups C and D for eight months.

RESULTS

The effects of the different diets on the teeth of these rats are given in Table III.

TABLE III

GROUP	NO. MONTHS ON DIET	NO. OF RATS	NO. WITH CARIES	NO. DECAYED TEETH	LOWER	UPPER
A	20	20	0	0	0	0
B	20	20	0	0	0	0
C	11	7	4	15	14	1
D	11	11	8	21	15	6
E	8	7	4	7	7	0
F	8	7	4	7	6	1

A typical decayed lower molar is shown in Fig. 4.

DISCUSSION

The rats in groups A and B did not develop caries after twenty months. This was probably due to the fact that their teeth were caries resistant. The results in the other four groups in which a number of rats developed caries show that caries susceptibility can be produced in these rats by pre- and post-natal feeding of high sugar diets. I obtained similar results in 1949 with white

rats.[2] The difference in the number of cavities produced in Groups C and E and in Groups D and F appears to be the question of time. Groups C and D were three months longer on the diets than Groups D and F. The difference in the number of cavities in the rats fed the coarse corn meal and those fed the high sugar diet is not significant. This suggests that the coarse corn particle which initiates caries in the white rat is not a factor in causing caries in these rats. The main contributing causes appear to be the caries-susceptible structure of the teeth and the high sugar content of the diet.

SUMMARY

1. A suitable rat, *Mystromys albicaudatus* has been used for the first time in South Africa in connection with dental caries research.

2. Caries could at first not be produced in that rat, when fed a high sugar diet for twenty months.

3. When female rats were fed a high sugar diet during pregnancy and lactation, caries could be produced in their offspring, when they were also fed a high sugar diet.

4. The coarse corn particle was not necessary to initiate caries in this rat.

I wish to express my thanks to the secretary for health, Dr. J. J. du Pre le Roux, for permission to submit this article for publication and to Miss H. L. Johnston, Mr. D. H. S. Davis, and Dr. H. le Riche for their generous and valuable assistance.

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EFFECT OF PARENTERAL ADMINISTRATION OF ANTIBIOTICS ON BACTERIAL POPULATION OF THE MOUTH

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THIS is one of a series of studies on human saliva as affected by antibiotics. The saliva is the point of interest in these studies because bacteremias following extraction of teeth have their origin in the mouth; the trauma of extraction permits organisms in the gingival sulcus to enter the blood stream. Prophylaxis consists in preoperative administration of antibiotics by parenteral injection.

In an earlier study,[1] it was shown that parenterally administered antibiotics are excreted in the saliva; the maximum concentrations of penicillin and streptomycin in the saliva are found about one hour after injection; the maximum concentration of chloramphenicol after one-half hour.[2] The question arises as to whether the levels of antibiotic found in the saliva are sufficient to affect the bacterial population of the mouth which in turn affect the bacteremia, or whether it is only the antibiotic present in the blood which is effective.

Two previous investigations have a bearing on this question, although they do not concern themselves directly with saliva. Eagle[3, 4] has shown that bacteria, particularly members of the viridans group, which have recovered from the toxic effects of penicillin, are extraordinarily susceptible to the action of the body's defense mechanism. This can be interpreted to mean that sublethal concentrations of antibiotics in the saliva may nevertheless be a factor in the final destruction of the bacteria elsewhere in the body.

The following is a study of the effects of penicillin, streptomycin, chloramphenicol, and a combination of penicillin and streptomycin on the bacterial population of the mouth after a single parenteral injection administered in accordance with the usual therapeutic practice.

PROCEDURE

Patients were given single doses of antibiotic parenterally. Saliva samples were collected before injection and also at the optimum level after injection. Bacterial counts were made on the saliva samples taken before and after injections. Antibiotic excreted into the saliva was assayed. Organisms were isolated from blood samples taken from patients having postextraction bacteremia; these organisms were tested in vitro for additive or synergistic effects of penicillin and streptomycin.

Administration of Antibiotics.—Patients were selected who had not been on prior antibiotic therapy for at least ten days.

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The antibiotics used, and their dosages, were as follows: (1) 400,000 units of penicillin (100,000 crystalline sodium penicillin and 300,000 procaine penicillin), (2) 1 Gm. of dihydrostreptomycin, (3) 1 Gm. of chloramphenicol in acetyldimethylamine, (4) a mixture of 1 Gm. of dihydrostreptomycin and 400,000 units of penicillin (100,000 crystalline sodium penicillin and 300,000 procaine penicillin). The antibiotics were administered intramuscularly except for chloramphenicol, which was given intravenously.

Ten patients were each given a single dose of the penicillin; another group of ten patients received single doses of the streptomycin; a third group, consisting of twenty patients, received single doses of chloramphenicol; and a fourth group of twenty received single doses of the penicillin-streptomycin mixture.

Collection and Preliminary Treatment of Saliva.—In order to induce salivation, patients chewed one-half inch paraffin blocks for three minutes. A 5 ml. sample of saliva was collected from each patient before injection; another 5 ml. sample was collected one hour after injection of penicillin, streptomycin, or penicillin-streptomycin mixture, or one-half hour after injection of chloramphenicol.

The saliva specimens, collected in clean, sterile sputum bottles, were frozen immediately in solid carbon dioxide and 95 per cent ethyl alcohol. They were stored at –15° C. until the following day, when they were defrosted at room temperature. A 0.1 ml. sample of saliva was withdrawn from each bottle for bacterial counts; the remainder was centrifuged at 1500 r.p.m. for fifteen minutes. The supernatant liquid was used for antibiotic assay.

Assay of Antibiotics.—Antibiotics were assayed by the turbidimetric method.[1, 5, 6]

The streptomycin assay made use of Oregon J. strain of *Staphylococcus aureus,* American type culture collection No. 9801, as the test organism. Streptomycin was assayed in the presence of penicillin by adding penicillinase to inactivate the penicillin.

The penicillin assay made use of FDA strain 209 *Staphylococcus aureus* as the test organism. The penicillin was assayed in the presence of streptomycin by rendering the test organism resistant to 100 Gm. of streptomycin through a process of multiple transfers to increasing amounts of streptomycin.

Staphylococcus aureus 209 was used in chloramphenicol assay.

Bacterial Counts.—A 0.1 ml. specimen of saliva was diluted with 0.9 ml. of a brain heart infusion broth containing 2.5 per cent of penicillinase and 0.033 per cent of liquoid (sodium polyanetholsulfonate). The liquoid was used to match its use in previous blood culture investigation,[7] where its function was to counteract the natural antibacterial activity of body fluids and to isolate that part of the antibacterial activity due solely to the antibiotics.

Specimens were diluted using a virus technique. The 10^{-4} or 10^{-5} dilutions were found to be most suitable for the gram-positive and negative counts. The coliform rods were lost at that dilution. Two 0.1 ml. samples of the appropri-

ately diluted material were transferred by means of a 0.2 ml. pipette to two blood agar plates and were spread on the plates by means of a glass spreader. Plates were incubated at 37° C. for eighteen hours, one of them under anaerobic conditions, the other under aerobic conditions, using 10 per cent carbon dioxide. Colonies were counted and the number of organisms per milliliter calculated as follows:

No. organisms per ml. = No. colonies on plate × dilution × 10.

To count fecal streptococci, undiluted specimens were heated to 60° C. for thirty minutes and then plated as just described. Any streptococci found on these plates after incubation under 10 per cent carbon dioxide were transferred to 6.5 per cent sodium chloride broth for confirmatory test.

In Vitro Studies on Effects of Antibiotics.—Cultures were made of blood taken from patients who had developed bacteremia after tooth extraction. Five different organisms isolated from different blood culture flasks were tested for additive or synergistic effects of penicillin and streptomycin. The antibiotics were estimated by the turbidimetric assay method.

Organisms were tested separately for sensitivity to penicillin and streptomycin by tube dilution technic. Each antibiotic titration was run in 1 ml. of medium to which 1 ml. inoculum was added and the final 2 ml. volume was incubated at 37° C. for eighteen hours. The inoculum was an eighteen-hour brain heart infusion broth culture which was diluted so that there were approximately 600,000 organisms per tube. End point was taken as the lowest concentration of antibiotic in which no visible growth occurred in eighteen hours. A control, containing no antibiotic but the same inoculum, was included each time.

Once minimum inhibitory concentration was thus established, subsequent titrations were run with penicillin to which fixed amounts of streptomycin were added. Usually one-fourth or one-half of the minimum inhibitory concentration of streptomycin was used. Thus, it was possible to determine penicillin sensitivity of a given organism in the presence of fixed amounts of streptomycin.

TABLE I

THE EFFECT OF A SINGLE INTRAMUSCULAR INJECTION OF PENICILLIN ON THE BACTERIAL POPULATION OF SALIVA

PATIENT	NO. OF ORGANISMS PER ML. OF SALIVA × 10^6				PENICILLIN IN SALIVA (UNITS/ML.)
	BEFORE PENICILLIN		1 HOUR AFTER PENICILLIN		
	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC	
1. S. T.	26.0	32.0	20.0	23.0	0.010
2. I. B.	19.0	23.0	17.0	30.0	0.023
3. S. M.	27.0	28.0	34.0	28.0	0.018
4. A. M.	8.5	120.0	8.0	30.0	0.006
5. C. M.	55.7	45.0	68.0	102.0	0.008
6. S. Ta.	69.0	62.0	58.0	54.0	0.013
7. D. D.	73.0	107.0	112.0	88.0	0.014
8. E. S.	24.3	31.0	17.6	25.0	0.013
9. J. D.	34.8	60.0	58.9	93.0	0.013
10. S. R.	224.0	201.0	154.0	142.5	0.020
Mean	56.1	70.9	54.2	61.5	0.014

RESULTS

Effect of Penicillin on Bacterial Count of Saliva.—One hour after a single intramuscular injection of 400,000 units of penicillin, the arithmetical mean count of aerobic bacteria in the saliva had changed from a pre-injection value of 56.1×10^6 to a value of 54.2×10^6; the mean anaerobic count changed from 70.9×10^6 to 61.5×10^6 (Table I). The variation is probably within the range of experimental error. There is no significant reduction in bacterial population count one hour after injection of a single dose.

The mean concentration of penicillin in saliva was 0.014 unit slightly less than the 0.030 unit found in the saliva after injection of a penicillin-streptomycin mixture (Tables I and IV).

Effect of Dihydrostreptomycin on Bacterial Count of Saliva.—One hour after a single intramuscular injection of 1 Gm. of dihydrostreptomycin, the mean count of aerobic bacteria in the saliva had changed from a pre-injection value of 29.92×10^6 to 39.36×10^6; the mean anaerobic count changed from 31.83×10^6 to 37.88×10^6 (Table II). The higher mean average after the administration is affected by two cases, Case 8 and Case 9. Possibly these patients had smoked or eaten not long before the control sample of saliva was taken, thereby reducing the initial bacterial count. Even if these two cases are not excluded, the variations in the before-and-after mean averages are within the range of experimental error. The conclusion is that streptomycin has no effect on the bacterial population count of the mouth one hour after injection of a single dose.

TABLE II

EFFECT OF A SINGLE INTRAMUSCULAR INJECTION OF 1 GM. DIHYDROSTREPTOMYCIN SULFATE ON BACTERIAL POPULATION COUNT OF SALIVA

	NO. OF ORGANISMS PER ML. OF SALIVA $\times 10^6$				
	BEFORE DIHYDROSTREPTOMYCIN		1 HOUR AFTER DIHYDROSTREPTOMYCIN		STREPTOMYCIN IN SALIVA (μG./ML.)
PATIENT	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC	
1. T. H.	11.0	17.0	6.0	32.0	2.8
2. M. H.	21.0	60.0	26.0	44.0	2.8
3. R. P.	3.5	5.0	2.1	1.9	2.5
4. D. H.	37.0	28.0	20.0	1.9	2.5
5. D. G.	16.7	17.0	27.0	35.0	2.5
6. W. S.	69.0	41.0	53.2	64.0	3.0
7. A. W.	61.0	72.3	67.0	43.0	3.6
8. G. M.	59.1	69.0	133.3	92.0	2.5
9. A. H.	6.7	10.0	44.0	50.0	4.2
10. J. F.	14.2	9.0	15.0	15.0	2.5
Mean	29.92	31.83	39.36	37.88	2.89

Effect of Chloramphenicol on Bacterial Count of Saliva.—One-half hour after a single intravenous injection of 1 Gm. of chloramphenicol, the mean count of aerobic bacteria in the saliva had changed from a pre-injection value of 42.0×10^6 to a value of 25.5×10^6; the mean anaerobic count changed from 42.6×10^6 to 30.3×10^6 (Table III). The aerobic population count was reduced about 40 per cent; the anaerobic count was reduced about 30 per cent.

TABLE III

THE EFFECT OF A SINGLE INTRAVENOUS INJECTION OF 1 GM. OF CHLORAMPHENICOL (IN ACETYLDIMETHYLAMINE) ON THE BACTERIAL POPULATION OF SALIVA

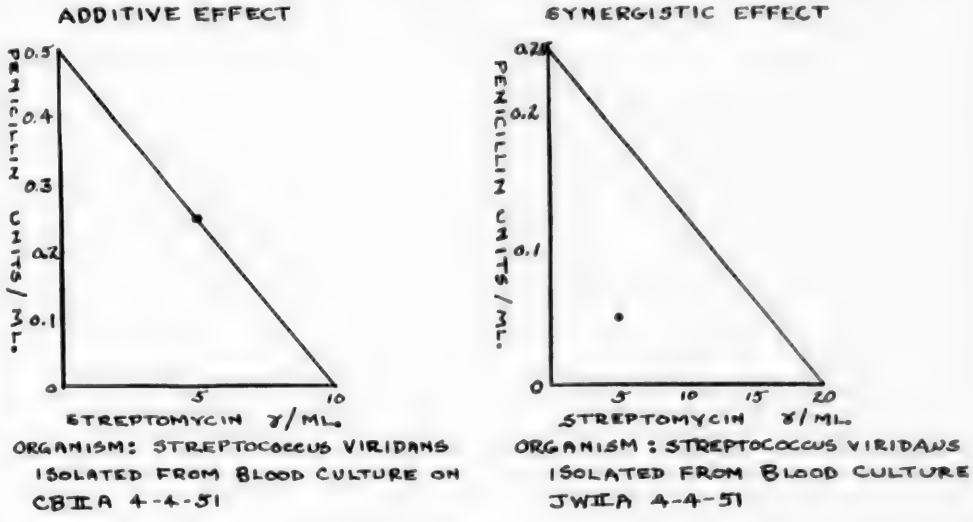
PATIENT	NO. OF ORGANISMS PER ML. OF SALIVA × 10^6				CHLORAMPHENICOL IN SALIVA (μG./ML.)
	BEFORE CHLORAMPHENICOL		½ HOUR AFTER CHLORAMPHENICOL		
	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC	
1. C. W.	133.0	104.0	144.0	195.0	2.3
2. A. S.	38.0	57.0	40.0	60.0	1.0
3. F. W.	35.0	48.0	17.2	72.0	1.0
4. F. W.	40.0	45.0	19.8	27.0	1.5
5. R. C.	21.0	39.0	16.5	20.0	–
6. B. B.	40.0	27.0	21.0	24.0	3.5
7. C. U.	50.8	47.0	34.0	15.0	3.5
8. J. E.	34.7	30.0	15.2	12.0	2.9
9. H. P.	7.0	18.0	4.0	3.0	1.9
10. V. U.	60.0	22.0	5.0	4.0	1.3
11. E. L.	33.0	13.0	30.0	10.0	0.9
12. J. E.	27.0	35.0	22.2	15.0	1.9
13. F. S.	13.5	30.0	15.0	33.0	1.5
14. A. B.	18.0	21.01	13.1	11.0	2.0
15. P. W.	35.0	31.0	27.7	16.0	1.0
16. J. G.	5.6	11.0	14.6	21.0	1.6
17. H. C.	67.0	51.0	22.1	26.0	1.2
18. C. W.	128.7	178.0	17.0	18.7	2.0
19. P. W.	28.6	21.0	22.9	12.3	1.2
20. M. S.	25.4	24.0	8.7	12.3	2.0
Mean	42.0	42.6	25.5	30.3	1.8

TABLE IV

THE EFFECT OF A SINGLE INTRAMUSCULAR INJECTION OF PENICILLIN AND STREPTOMYCIN ON THE BACTERIAL POPULATION OF SALIVA

PATIENT	NO. OF ORGANISMS PER ML. OF SALIVA × 10^6				PENICILLIN IN SALIVA (UNITS PER ML.)	STREPTOMYCIN IN SALIVA (μG./ML.)
	BEFORE PENICILLIN & STREPTOMYCIN		1 HR. AFTER PENICILLIN & STREPTOMYCIN			
	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC		
1. R. M.	30.0	29.0	6.8	6.7	0.030	1.6
2. C. F.	143.0	147.0	17.0	47.0	0.030	2.8
3. L. M.	7.0	4.9	9.0	12.0	0.031	2.3
4. R. C.	17.0	56.0	7.0	8.0	0.034	2.4
5. W. B.	20.0	26.0	6.4	22.0	0.0125	1.1
6. E. W.	36.0	33.7	11.0	18.0	0.024	2.0
7. D. C.	16.0	13.0	7.0	8.0	0.028	3.2
8. A. P.	53.4	46.0	14.0	15.0	0.024	3.8
9. L. B.	79.5	74.0	48.0	34.0	0.029	2.3
10. T. P.	26.7	22.0	35.0	30.0	0.029	2.2
11. P. C.	19.0	38.2	8.0	10.0	0.028	3.6
12. T. P.	9.0	41.0	10.0	10.0	0.030	4.0
13. E. C.	44.0	50.0	26.0	10.0	0.027	3.6
14. L. B.	49.0	56.0	12.0	14.0	0.034	4.0
15. A. B.	37.5	57.0	7.0	7.0	0.030	3.0
16. C. U.	12.4	13.2	14.0	11.0	0.0125	2.0
17. N. T.	80.0	75.0	10.0	2.1	0.017	2.0
18. G. W.	18.0	28.0	8.0	7.5	0.058	2.5
19. T. T.	26.0	14.0	3.0	4.0	0.062	2.3
20. I. G.	12.0	20.0	5.3	5.2	0.040	4.0
Mean	37.2	42.2	13.3	13.0	0.030	2.7

Effect of Penicillin-Streptomycin Mixture on Bacterial Count of Saliva.—One hour after a single intramuscular injection of a mixture of 1 Gm. of dihydrostreptomycin and 400,000 units of penicillin, the mean count of aerobic bacteria in the saliva had changed from a pre-injection value of 37.2×10^6 to a



STREPTOMYCIN SENSITIVITY
IN PRESENCE OF PENICILLIN

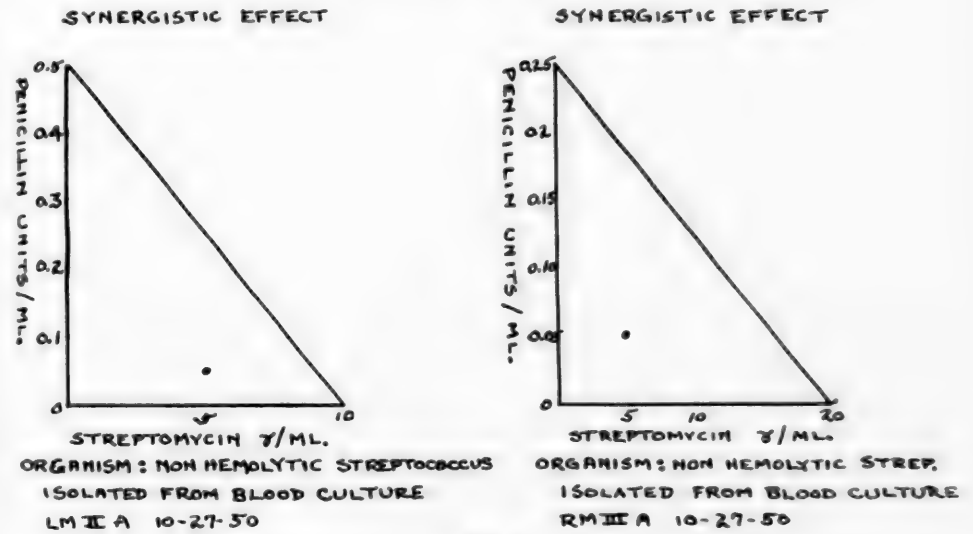


Fig. 1.

value of 13.3×10^6; the mean anaerobic count changed from 42.2×10^6 to 13.0×10^6 (Table IV). The aerobic and the anaerobic counts were each reduced about 65 per cent. This reduction may be attributed to the increase in total antibiotic excreted in the saliva and also to synergistic action against organisms found in the mouth. In vitro experiments justify the assumption of synergism.

Detailed consideration of the data shows that in one instance (Case 16) the antibiotic mixture had practically no effect; in two instances (Cases 3 and 10) there was a rise in the number of organisms in spite of antibiotic concentrations in the saliva not far from the average. These deviations can be explained variously as due to experimental error, to the stimulating effect of antibiotics on certain organisms, or to the fact that when organisms are depressed to a certain low critical level in the mouth there is a sudden compensatory rise in the number of organisms. (This last phenomenon has been observed occasionally, particularly when antibiotics are applied locally, as in troches.)

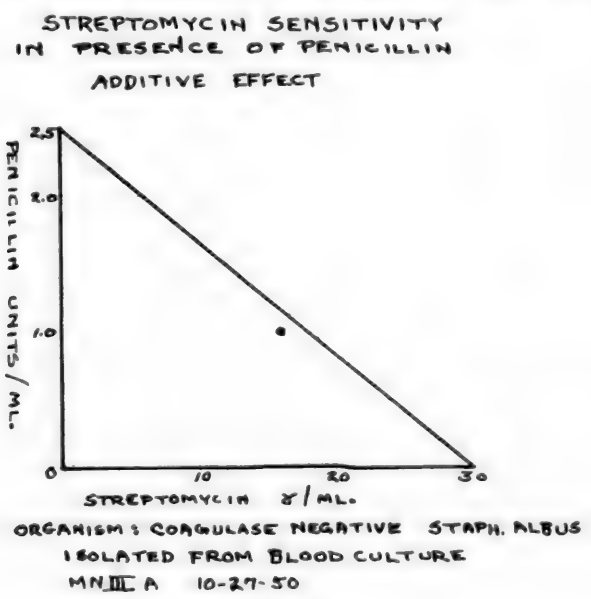


Fig. 2.

Concentration of streptomycin in saliva was about the same whether it was administered by itself (2.7 μg streptomycin per milliliter of saliva) or in a mixture with penicillin (2.89 μg streptomycin per milliliter saliva). Concentration of penicillin in saliva was actually less when the penicillin was administered by itself (0.014 unit penicillin per milliliter saliva) than when it was administered in the mixture (0.030 unit penicillin per milliliter saliva). The latter figure is reliable, checking favorably with results from another study[1] in which the average for forty-four cases was 0.029 unit penicillin per milliliter saliva. The 0.014 value is less reliable, being based on only ten cases.

In Vitro Effects of Penicillin-Streptomycin Mixture.—The effects of penicillin-streptomycin mixture on organisms isolated from the blood of patients with postextraction bacteremia are recorded on triangle diagrams (Figs. 1 and 2). Data are for fixed quantities of the organisms.

The bacteriostatic doses of antibiotics for the *Streptococcus viridans* organisms isolated from blood culture CBIIA are shown (Fig. 1) to be as follows:

penicillin alone	0.5 unit per ml.
streptomycin alone	10 μg per ml.
penicillin-streptomycin	0.25 penicillin unit per ml. and 5 μg streptomycin per ml.

This constitutes an additive effect as shown arithmetically, only half as much penicillin and half as much streptomycin being needed in the mixture, and as shown graphically, the three values falling on a straight line.

For *Streptococcus viridans* isolated from blood culture JWIIA, the bacteriostatic doses are:

penicillin alone	0.25 unit per ml.
streptomycin alone	20 μg. per ml.
penicillin-streptomycin	0.05 penicillin unit per ml. and 5 μg. streptomycin per ml.

This constitutes a synergistic effect, as less than half of each antibiotic is needed in the mixture. The value for the mixture falls inside the triangle.

Similarly, synergistic effects are shown for the nonhemolytic streptococci isolated from blood cultures LMIIA and RMIIIA, and an additive effect is shown for the coagulase negative *Staphylococcus albus* isolated from blood culture MNIIIA.

SUMMARY AND CONCLUSIONS

1. The effects of various antibiotics, administered parenterally, on the bacterial population of the saliva were measured; these effects were studied because bacteria in the mouth are the source of the bacteremia which follows tooth extraction.

2. Varying portions of the penicillin, streptomycin, chloramphenicol, or penicillin-streptomycin mixture injected parenterally were secreted into the saliva.

3. Neither a single parenteral dose of penicillin nor a single parenteral dose of streptomycin had any significant effect on the bacterial population of the saliva.

4. A single parenteral dose of chloramphenicol depressed the aerobic population of the saliva about 40 per cent and the anaerobic population about 30 per cent.

5. A single parenteral dose of a penicillin-streptomycin mixture depressed the aerobic population of the saliva about 65 per cent; the anaerobic population likewise was depressed about 65 per cent.

6. In vitro tests showed that penicillin and streptomycin have additive and/or synergistic effects against five strains of organisms isolated from the blood stream following postextraction bacteremia. This may explain the in vivo effectiveness of penicillin-streptomycin mixture in reducing the bacterial population of the saliva.

7. The advantages of administering penicillin-streptomycin mixture parenterally before extraction of teeth are (a) 65 per cent of the organisms in the mouth are inhibited by the combined action of the penicillin and streptomycin excreted in the saliva, (b) organisms surviving in the mouth may (according to Eagle) be so weakened that they easily succumb elsewhere in the body to the natural body defenses, and (c) organisms still surviving in the blood stream are still subject to systemic antibiotic attack.

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CHEMICAL STUDIES IN PERIODONTAL DISEASE

X. PAPER CHROMATOGRAPHIC INVESTIGATION OF THE PUTREFACTION ASSOCIATED WITH PERIODONTITIS

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PERIODONTAL disease has been the subject of a tremendous number of articles in the dental literature. Most of these studies have been undertaken from a clinical, histologic, or bacteriologic viewpoint. Biochemical studies with a firm scientific foundation are, in comparison, scarce. The result is a plethora of information, much of it of high quality, but there is no generally accepted theory to provide a rationale for the prevention and treatment of periodontal disease. It is to be hoped that a biochemical approach to the problem will have some success as it has in many other diseases, including dental caries. It was for this reason that the present series of investigations was undertaken.

That the confusion is real is evidenced by the large number of names that have been, and still are, applied to the several forms of periodontal disease. The term "periodontitis" will be used here to represent that disease, or perhaps group of diseases, characterized by periodontal pocket formation, alveolar bone resorption, and inflammatory suppuration. Though this may be an oversimplification, it is necessary due to the lack of distinguishing diagnostic procedures.

There is no question but that protein destruction accompanies periodontitis. The evidence that this may be described, at least in part, as putrefaction is considerable. It has been shown that odor production, formation of indole and sulfides, and liberation of free amino groups (formol titration) proceeds more rapidly in salivas from patients with periodontitis than those without periodontal disease.[1, 2, 3] This may be considered with respect to the following simplified picture of protein putrefaction.

Protein → Amino Acids → Putrefactive End Products

Odor, indole, sulfides, and possibly free amino groups are representative of putrefactive end products. It is then of interest to study another part of the above scheme, namely the amino acids. This is the purpose of the present study. As will be seen, it will also be possible to gain further insight into the whole putrefactive process.

The method chosen for this investigation is paper chromatography.[4] The separation of substances by this procedure is the result of different distributions between a stationary paper support and a developing solvent which is allowed to move over the paper. The positions of a wide variety of amino

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compounds, of which amino acids are most commonly found, are revealed by treatment with ninhydrin. Identification is possible since the position of a substance relative to its neighbors is unique, or nearly so.

METHODS AND MATERIALS

Collection of Salivas.—Patients were selected from those entering the dental clinic by the following criteria: (1) the presence of at least several suppurating periodontal pockets, (2) bone resorption as evidenced by radiographs, (3) no recent periodontal treatment, and (4) either no breakfast on the day of saliva collection or the absence of sugar from the breakfast. Normal salivas were obtained from dental students free of any suspicion of periodontal disease and with the requirement concerning breakfast. Approximately 20 ml. of saliva were collected from each donor by paraffin stimulation between the hours of 9 and 9:30 A.M.

Preparation and Incubation of Salivas.—Immediately after collection was complete, 4 ml. were withdrawn and run into 16 ml. of 95 per cent ethanol to stop all enzymatic activity and precipitate protein. These samples constituted "fresh"* salivas. Two 5 ml. portions of the original saliva were placed in separate 20 ml. vials containing 50 mg. of casein. The vials were closed and incubated on a rotating drum in a water bath at 37° C. One vial was removed after 2 hours and the other after 7 hours. When incubation was complete, 4 ml. were withdrawn and run into ethanol as before. The alcoholic samples were allowed to stand in the refrigerator for 6 to 8 days. The precipitated protein was removed by centrifugation and the supernatant was freed of salts by the ion exchange method previously described.[5] The desalting removed a large part of all substances that were not amphoteric. The recovery of amino acids was complete within the experimental error of the chromatographic procedure with the exception of arginine which was almost completely lost.[5]

Ten normal and 7 pathologic salivas were prepared in this way. In addition, 1 normal and 1 pathologic saliva were carried through the same procedure except that casein was omitted.

Three pathologic and 4 normal salivas were hydrolyzed by adding 1 ml. of 12N HCl to 1 ml. of whole saliva and heating in a sealed tube at 100° C. for 24 hours. The acid was removed by several concentrations to dryness in a vacuum desiccator. The residue was made up to the original 1 ml. with water.

One-dimensional Paper Chromatography.—The techniques employed were essentially those of Bull, Hahn, and Baptist,[6] except that longer strips of paper and different solvents were used. In the case of "fresh" and incubated salivas, 1 ml. of the alcoholic solutions, prepared as described above, were concentrated directly on paper strips,[7] 12 cm. from one end, of Eaton-Dikeman No. 613 cut 7 mm. wide and 90 cm. long. For hydrolyzed salivas, 20.4 μl was applied with a micropipette in portions of 2 to 3 μl. Approximately 3 μl of 30 per cent hydrogen peroxide was applied to the sample on each strip. This treatment converted cystine and cysteine to cysteic acid and methionine to methionine sulfone.[8]

*The quotation marks are used to distinguish this type of saliva, collected by expectoration, from saliva obtained as it is formed in the salivary glands and immediately stabilized.

The strips were developed by the descending method either with n-butanol-acetic acid-water, 4:1:2 by volume (designated solvent BA) or 2,4-lutidine-water, 3:2 by volume (designated solvent L). Solvent BA was allowed to run over the strips for 42 to 44 hours and solvent L for 66 to 68 hours. In these periods the solvent traversed the full length of the strip and dripped from the ends. This allowed the full length of the strip to be utilized. The chromatographic chambers were kept in a constant temperature room at 29.5 ± 0.5° C.

Inasmuch as the solvents were allowed to pass the ends of the strips, the usual R_f value could not be used to designate the position occupied by a given amino acid. To provide a reference point, approximately 2 μl of 0.05M α-aminoheptoic acid was applied to each strip. This amino acid traveled about 70 cm. ahead of all others. The position occupied by an amino acid was defined by a value, designated R_H, as follows:

$$R_H = \frac{\text{distance moved by a given amino acid}}{\text{distance moved by } \alpha\text{-aminoheptoic acid}} \; 100$$

R_H values for a number of amino acids, determined by chromatography of known solutions, are given in Table I.

TABLE I. R_H VALUES*

AMINO ACID	ABBREVIATION	SOLVENT BA	SOLVENT L
1. alanine	ALA	49	45
2. β-alanine	β-ALA	50	36
3. α-amino-n-butyric acid	α-BUT	62	57
4. γ-aminobutyric acid	γ-BUT	60	37
5. δ-amino-n-caproic acid	δ-CAP	80	42
6. ϵ-aminocaproic acid		80	38
7. α-amino-n-heptoic acid	H	100	100
8. δ-aminovaleric acid	δ-VAL	70	37
9. arginine	ARG	26	17
10. asparagine		28	29
11. aspartic acid	ASP	33	22
12. citrulline		35	35
13. cysteic acid	CYS	15	47
14. γ-ethylglutamate	ET GLU	80	80
15. glucosamine	GLUC	28	80
16. glutamic acid	GLU	42	24
17. glutamine		35	35
18. glycine	GLY	35	35
19. histidine	HIS	22	42
20. hydroxyproline	HO-PRO	40	46
21. isoleucine	LEUS {ISOLEU	88	85
22. leucine	{LEU	90	88
23. lysine	LYS	21	11
24. methionine		76	84
25. methionine sulfone		35	68
26. methionine sulfoxide		35	40
27. norleucine		91	91
28. ornithine		20	12
29. phenylalanine	ϕ-ALA	85	90
30. proline	PRO	55	50
31. serine	SER	35	40
32. taurine		27	68
33. threonine	THR	43	46
34. tryptophane		74	94
35. tyrosine	TYR	63	94
36. valine	VAL	75	70

*$R_H = \frac{\text{distance moved by a given amino acid}}{\text{distance moved by } \alpha\text{-aminoheptoic acid}}$ 100. The values presented are not necessarily average values but are representative, chosen so as to accurately show the position of any amino acid to its near neighbors.

Two-dimensional Paper Chromatography.—The techniques used were essentially those of Williams and Kirby.[9] The paper was Whatman No. 1 cut 16.5 inches square. The sheets were delevoped first with solvent L and then with solvent BA, each for about 42 hours. The spots were identified by visual comparison of relative positions as suggested by Dent.[8]

Approximately half of the "fresh" and incubated saliva samples were chromatographed in this way, using 1.5 ml. of solution, to establish qualitative identification of the ninhydrin-reacting components. For whole saliva hydrolysates, 50 μl was applied to the sheet in portions of 2 to 3 μl. Peroxide treatment was used as before.

A composite two-dimensional chromatogram of known amino acids is presented in Fig. 1.

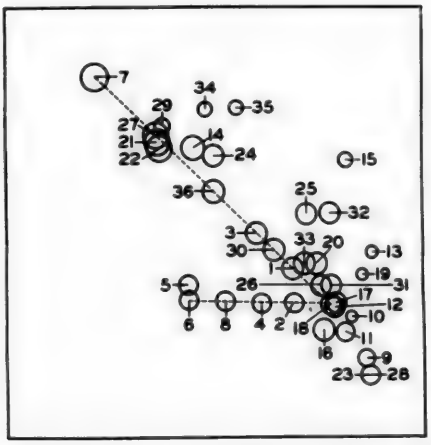


Fig. 1.—Composite, two-dimensional chromatogram of known amino acids. Amino acids were applied in lower right corner. Solvent L traveled vertically and solvent BA horizontally. See Table I for identification of the numbers.

Quantitation of Amino Acids.—The method of Bull, Hahn, and Baptist[6] was used. In this procedure, a one-dimensional chromatogram was pulled in steps of 0.5 cm. between a light source and a photocell. The transmittance at each step was recorded and plotted against the distance along the strip. Each colored band on the chromatogram appeared on the graph as a depression in the transmittance. The area of each depression was measured with a planimeter. This area provided a measure of the component(s) present in the band. If only one substance was present, its concentration could be determined by reference to a calibration curve. If two or more substances were present in a given band, it was not possible to express their concentrations separately, but they were compared as a group represented by total area with the corresponding band on other chromatograms.

The accuracy of this method was not great. The error of a single determination was often about 10 per cent and occasionally much larger, particularly if the area was small, less than about 50 planimeter units. For this reason, statistical evaluation of the data was especially important.

RESULTS

Whole Hydrolyzed Saliva.—A typical, two-dimensional chromatogram of a whole hydrolyzed saliva is shown in Fig. 3, *a*. All salivas, normal and pathological, gave similar chromatograms. The spots identified as tyrosine, histidine,

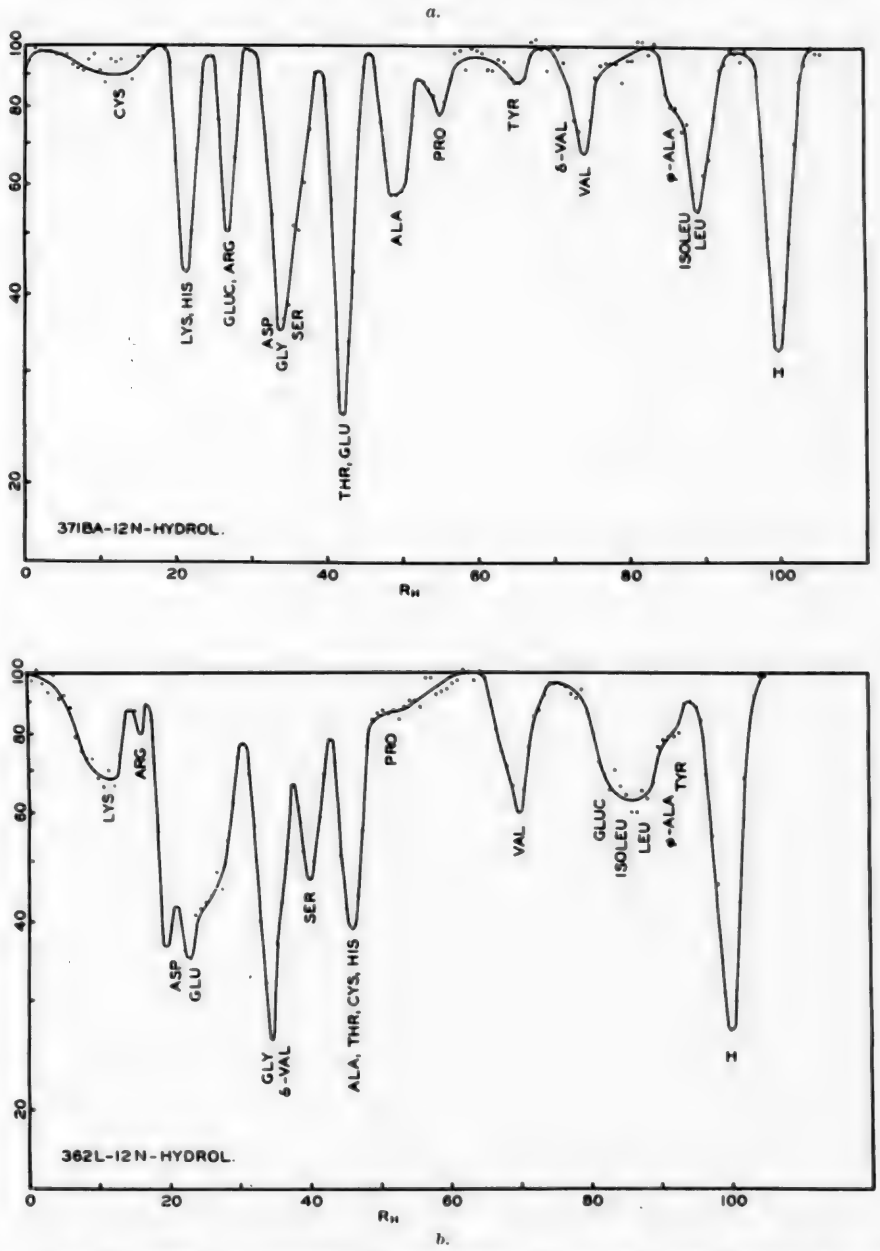


Fig. 2.—Scanned, one-dimensional chromatograms of a normal, whole hydrolyzed saliva: *a*, developed with solvent BA, *b*, developed with solvent L$_A$. See Table I for explanation of the abbreviations.

glucosamine and δ-amino-valeric acid(?) were always very dim and sometimes not visible, though probably always present. The amino acids identified include all those commonly found in proteins except methionine and hydroxy-proline. If either of these was present, it was so in very small amounts. The only substance that was not common to biological material was tentatively identified as δ-amino-valeric acid. It occurred free in saliva and presumably was not a protein constituent. It will be discussed later.

With qualitative identification available from two-dimensional chromatograms, it was possible to determine the components in each band on one-dimensional chromatograms. Two typical, scanned chromatograms appear in Fig. 2, *a* and *b*. Because of the complexity of the solutions, quantitation had to be confined mostly to a comparison of corresponding areas of groups of amino acids. Since no differences between hydrolyzed salivas from normal individuals and patients with periodontitis were observed, the numerical data are not included here except for the few amino acids which were sufficiently resolved for individual measurement. These appear in Table II.

TABLE II. CONCENTRATIONS OF SOME OF THE AMINO ACIDS IN WHOLE HYDROLYZED SALIVA

AMINO ACID	μM/ML. SALIVA*
cysteic acid†	0.32
tyrosine	0.30
alanine	1.01
valine	1.56
serine	1.79

*Each value is the average of determinations on seven different salivas.
†Originated from cystine and/or cysteine.

"Fresh" and Incubated Salivas.—Fig. 3, *b*, *c*, and *d* shows 3 typical two-dimensional chromatograms. A few scanned, one-dimensional chromatograms appear in Fig. 4, *a*, *b*, *c*, and *d*. Seven-hour-incubated salivas are not included in the interests of brevity since they were similar to the two-hour-incubated samples except that all bands were larger.

In "fresh" saliva it was possible to identify lysine, glycine, alanine, and a substance believed to be δ-aminovaleric acid. In addition, glutamic acid was always visible one-dimensionally, though not consistently apparent by the less sensitive two-dimensional technique. Traces of other ninhydrin-reacting substances were occasionally observed but in amounts too small to be identified. The small band following lysine on the one-dimensional chromatograms probably represented a small amount of arginine that escaped the desalting. Because of the loss of this amino acid, it was not possible to determine whether or not it was a major component.

In incubated salivas many of the common amino acids were identified. These are summarized in Table III together with the band designation used in Fig. 4. Qualitatively, there was very little individual variation.

One of the major components of both "fresh" and incubated salivas, normal and pathologic, was not a common amino acid. That it might be δ-aminovaleric acid was suspected when it was observed that it occupied the expected

position in the homologous series consisting of glycine, β-alanine, γ-aminobutyric acid, etc. This is illustrated in Fig. 3, *d*. Its position could not be distinguished from that of known δ-aminovaleric acid. However, such evidence can be considered only as an indication of identity when studying an uncommon substance. Further evidence was provided by the observation that both the known and the suspected amino acid exhibited an unusual behavior on one-dimensional chromatograms developed with solvent BA. Sometimes δ-aminovaleric acid was observed to overlap tyrosine (band 6, see Fig. 3, *c*) and at other times valine (band 8, see Fig. 3, *d*). This was proved to be the case with suspected substance by elution and rechromatography, in solvent L, of the

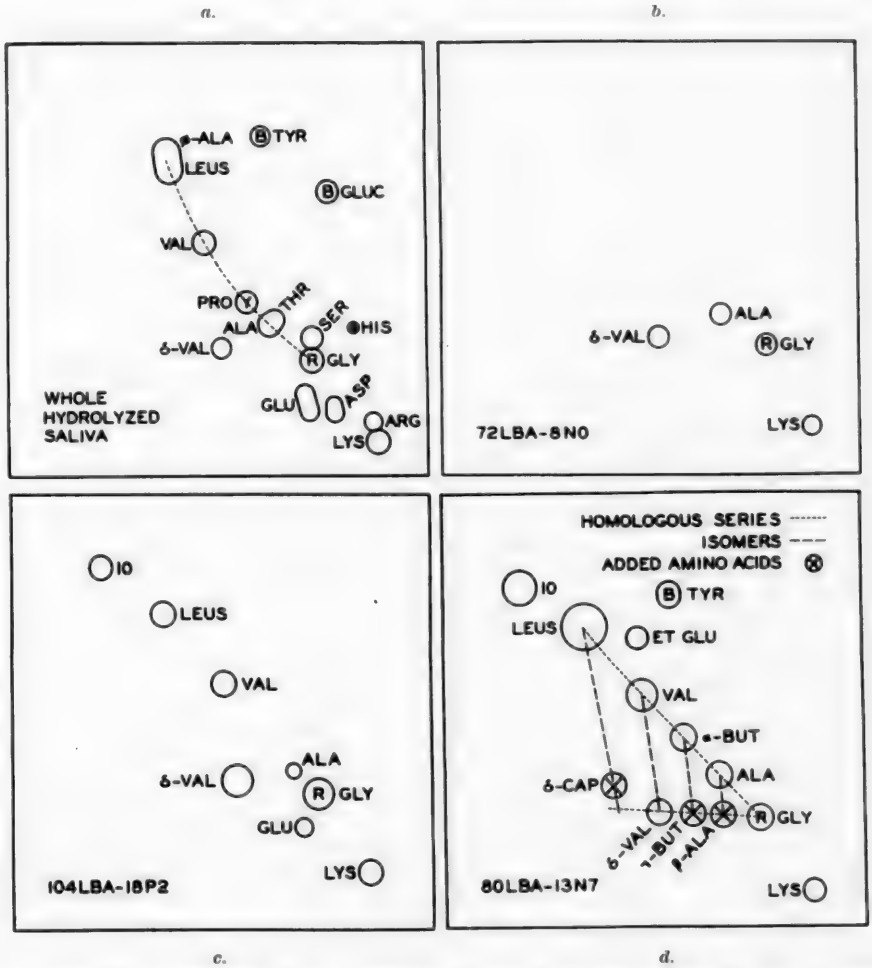


Fig. 3.—Two-dimensional chromatograms: *a*, whole hydrolyzed saliva, *b*, "fresh," normal saliva, *c*, two-hour-incubated, pathologic saliva, *d*, seven-hour-incubated, normal saliva. Solutions were applied in the lower right corner. Solvent L traveled vertically and solvent BA horizontally. *B* = brownish purple. *R* = reddish purple. *Y* = yellow. See Table I for explanation of the other abbreviations.

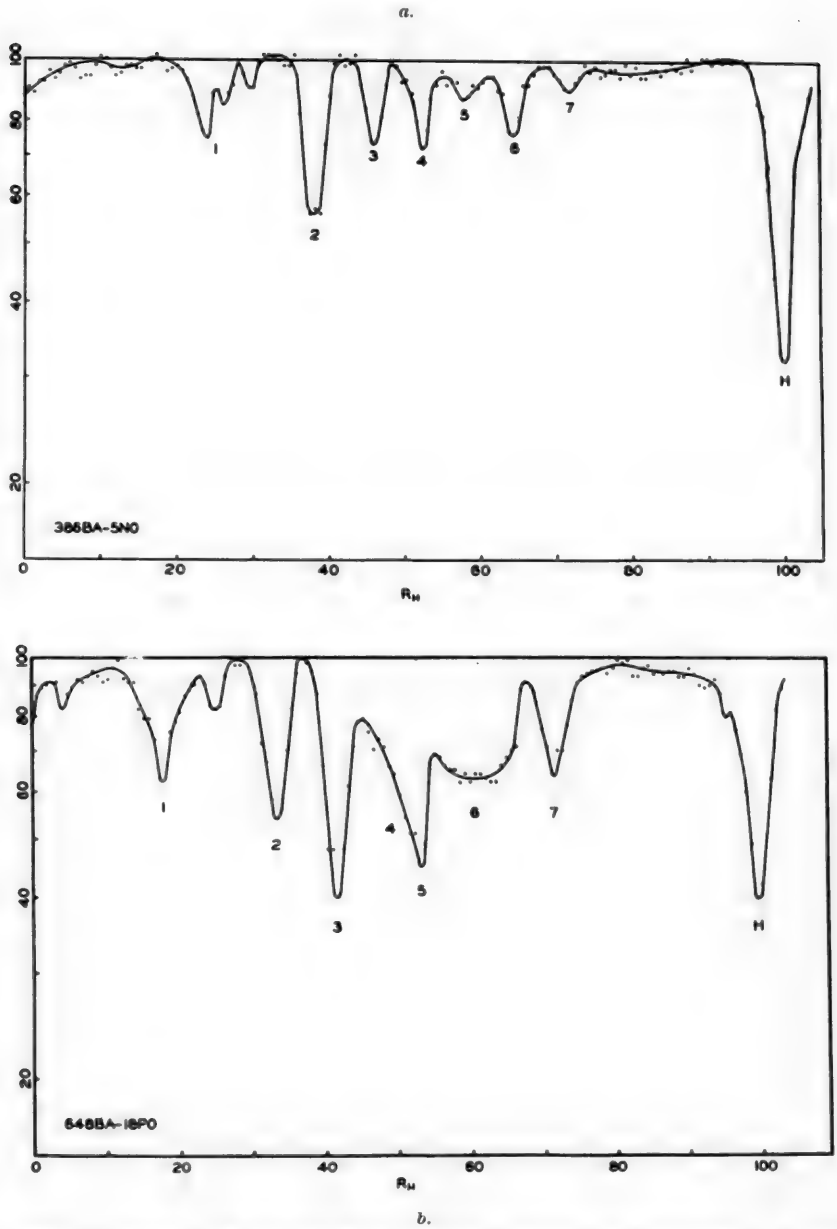
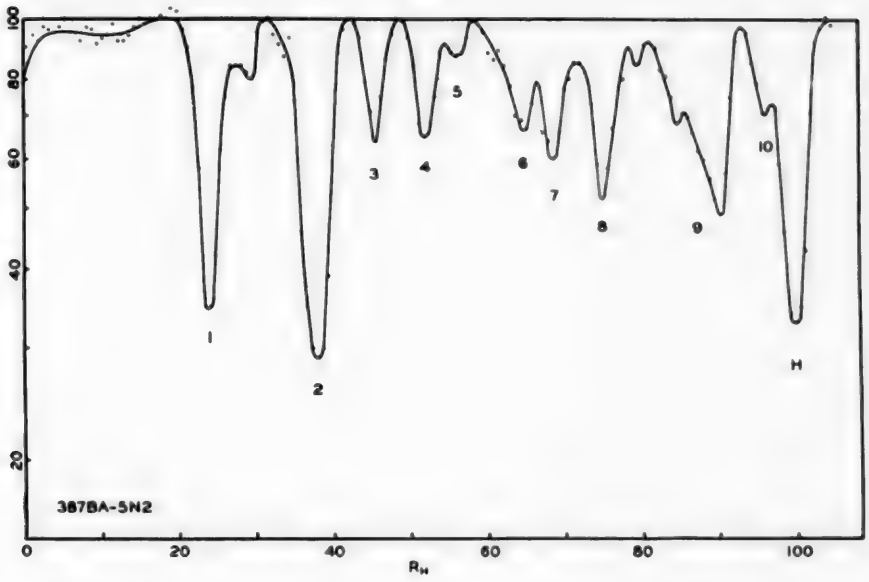
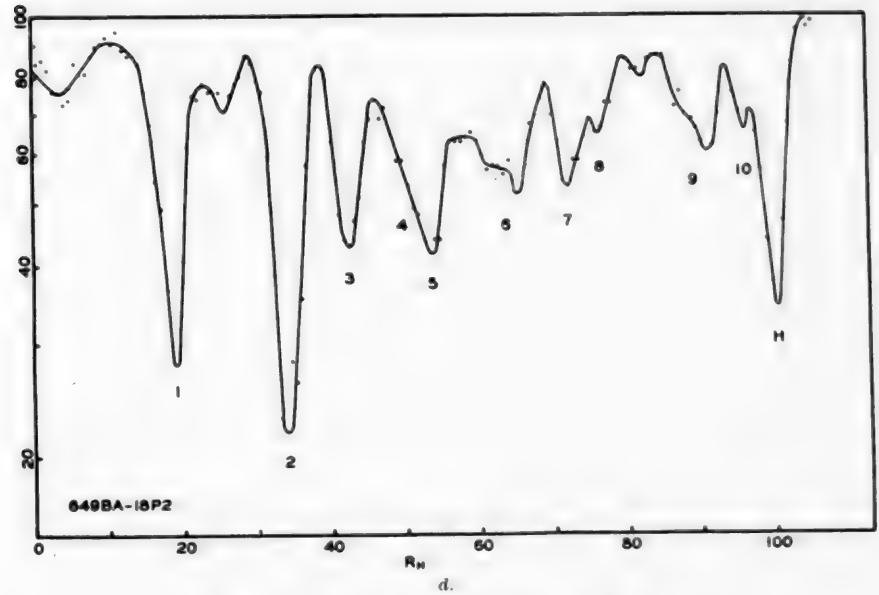


Fig. 4.—Scanned, one-dimensional chromatograms developed with solvent BA: *a*, "fresh," normal saliva, *b*, "fresh," pathologic saliva, *c*, two-hour-incubated, normal saliva, *d*, two-hour-incubated normal saliva. See Table III for identification of the band numbers. H = α-amino-heptoic acid added for reference.

c.





d.

Fig. 4.—*c* and *d*. *(For legends, see opposite page.)*

TABLE III. NINHYDRIN-REACTING SUBSTANCES IN "FRESH" AND INCUBATED SALIVAS

BAND DESIGNATION	MAJOR COMPONENTS	MINOR COMPONENTS	POSSIBLY PRESENT IN TRACES
1	lysine	peptides histidine	ornithine
2	glycine	peptides aspartic acid	serine
3	glutamic acid volatile amine	peptides	threonine
4	alanine	peptides	
5	volatile amine	peptides proline	
6	volatile amine tyrosine	peptides α-aminobutyric acid	
7	δ-aminovaleric acid (?)	peptides	
8	valine	peptides	tryptophane
9	leucine isoleucine	peptides phenylalanine	
10	unidentified	peptides	

material in sections cut from chromatograms of incubated salivas. The reason for this behavior is not known. Hydrogen ion concentration has a similar effect on certain amino acids, but this could not be shown to be the case here.

The presence of volatile amines was suspected when it was noted that more ninhydrin-reacting material was present on one-dimensional chromatograms than could be accounted for on the basis of spots which appeared on two-dimensional chromatograms. This was especially true of pathologic salivas. Volatile amines were suggested since they would have been lost from two-dimensional chromatograms during development with solvent L which provided basic conditions. This was proved to be the case by distillation from basic solution of a number of pooled, pathologic saliva samples remaining from the previous work. Chromatography of the distillate revealed that volatile amines were present in bands 3, 5, and 6. Probably two were present, one forming a double band (5 and 6) very like the behavior of methyl amine. They were not identified further since the chromatographic procedure was not optimum for their study. Further work is in progress.

The finding of volatile amines was at first somewhat surprising since the desalting was designed to remove all substances that were not amphoteric. It was apparent that the removal was not complete and that the amounts found were certainly less, and possibly much less, than were originally present.

The substance designated 10 was present in all incubated salivas and possibly in a few "fresh" samples. It did not survive hydrolysis with 6N HCl for 24 hours at 100° C. It may, therefore, have been a peptide.

Acid hydrolysis also produced approximately a twofold increase in amino acid concentrations, a general narrowing of all bands, and the disappearance of the color preceding band 1. Therefore, small amounts of peptides were present throughout the chromatograms, particularly in the region preceding band 1.

Band 1 on chromatograms of seven-hour-incubated salivas was usually very irregular and contained unidentified substances.

TABLE IV. CONCENTRATIONS* OF NINHYDRIN-REACTING SUBSTANCES IN "FRESH" AND INCUBATED SALIVAS

	HOURS INCUB.	Band No.†										
		1	2	3	4	4 + 5	5	6	6 + 7	7	8	9	10
N‡	0	52	80	28	39	71	32	61		34	0	0	0
σ§		16	27	16	16	27	14	33		15			
P‖	0	63	86	95		167		142		63	0	0	0
σ		26	38	16		41		58		13			
"t"¶				8.3		5.82		3.68		4.11			
"p"**				<0.01		<0.01		<0.01		< 0.01			
N	2	145	176	50	53	92	39		127		88	106	37
σ		43	50	32	15	29	31		40		37	52	23
P	2	159	161	105		169			271		61	106	30
σ		46	66	19		30			62		24	31	20
"t"				4.04		5.31			5.95				
"p"				<0.01		<0.01			< 0.01				
N	7	159	192	73	84	122	38		226		260	370	67
σ		55	52	41	32	34	25		71		59	86	22
P	7	230	211	145		258			316		264	367	69
σ		28	65	47		65			68		32	90	18
"t"		3.10		3.38		5.67			2.65				
"p"		<0.01		<0.01		<0.01			< 0.05				

*The concentrations are expressed as area (planimeter units) of the indicated band on scanned, one-dimensional chromatograms.

†See Table III for the components of each band.

‡Normal saliva. Each value is the average of determinations on ten different salivas.

§Standard deviation.

‖Pathologic saliva. Each value is the average of determinations on seven different salivas.

¶"t" = $\frac{\text{difference between the means}}{\text{standard error of the difference}}$.

**"p" = the probability that the difference between the means is due to chance.

Quantitation.—The areas of the major bands on the scanned, one-dimensional chromatograms were measured with a planimeter. The results are presented in Table IV. Since some of the bands overlapped, it was not always possible to obtain separate measurements. In these cases, the areas appear as

TABLE V. CONCENTRATIONS OF SOME OF THE AMINO ACIDS IN "FRESH" AND INCUBATED SALIVAS

	HOURS INCUBATED	μM/ml. SALIVA	
		NORMAL*	PATHOLOGIC†
Band 1—	0	0.07	0.09
lysine	2	0.37	0.45
Band 2—	0	0.20	0.22
glycine	2	1‡	1‡
	7	> 1‡	> 1‡
Band 3—	0	0.05	
glutamic acid	2	0.10	
	7	0.16	
Band 4—	0	0.06	
alanine	2	0.09	
	7	0.15	
Band 7—	0	0.13	0.28
δ-aminovaleric acid	(?)		
Band 8—	0	0	0
valine	2	0.31	0.20
	7	> 1‡	> 1‡

*Each value is the average of determinations on ten different salivas.

†Each value is the average of determinations on seven different salivas.

‡An approximate value obtained by extrapolation of the calibration curve.

a sum in the tables. The concentrations are given in planimeter units since many of the bands contained more than one component. Included are statistical values for those differences which are significant. The few areas that were known to contain only one major component were converted to concentration units by reference to calibration curves. These are presented in Table V. These values are probably all slightly high since every band contains one or more minor components.

Since the chromatograms of "fresh," normal salivas were relatively simple, all of the major ninhydrin-reacting components could be expressed in concentration units with the exception of the volatile amines, which were absent or nearly so, and arginine, which was largely lost during desalting.

Bands 3, 4 plus 5, and 6 plus 7 had smaller areas on chromatograms of normal salivas compared to pathologic salivas. These differences appeared in "fresh" and incubated salivas but remained essentially constant during incubation. A difference was also apparent in band 1, but only on chromatograms of seven-hour-incubated salivas. The significance was not apparent, since the components were not completely identified. All of these differences had a high degree of statistical significance. From the nature of the chromatograms it appeared probable that the differences in bands 3 and 4 plus 5 were due only to the volatile amines. Both a volatile amine and the substance believed to be δ-aminovaleric acid were probably responsible for the difference in band 6 plus 7. In other words, the observed differences between normal and pathologic salivas could not be ascribed to any of the common amino acids.

The chromatograms of the 2 salivas (1 normal and 1 pathologic) that were incubated without casein changed very little during incubation. The bands did not increase in area and bands 8, 9, and 10 did not appear. It was apparent that very little if any putrefaction had taken place. The presence or absence of casein was an important factor in this type of incubation.

DISCUSSION

Free Amino Acids in Saliva.—In this paper lysine, glycine, alanine, glutamic acid, and a substance which may be δ-aminovaleric acid are reported as the major free amino acids in saliva, with traces of at least some of the others. Kirch, Kesel, O'Donnell and Wach[10] using microbiological assay, and Goldberg, Gilda, and Fishkoff,[11] using two-dimensional paper chromatography, reported the presence of most of the common amino acids. The latter group also found an unidentified substance which they called "under alanine." Berry[12] reported the same common amino acids as found in this study plus serine and aspartic acid in some salivas. She also found an unidentified substance which was a major component. The most recent study of free amino acids, by Moor and Gilligan,[13] reported much the same results except that they found an uncommon amino acid which they identified as γ-aminobutyric acid on the basis of its position on a two-dimensional chromatogram. It is probable that the uncommon amino acid found in these three studies[11, 12, 13] and the substance reported here as

possibly being δ-aminovaleric acid are all the same. Berry[12] showed that it could not be γ-aminobutyric acid. The work in this paper indicates the same result. The final answer to the identity of this substance must await further work.

If it is indeed δ-aminovaleric acid, its presence is easily explained. It has been known for many years that δ-aminovaleric acid is a product of the putrefaction of ornithine, arginine, proline, or hydroxyproline.[14, 15, 16, 17] Since it has been shown here that the suspected δ-aminovaleric acid occurs in "fresh" saliva from patients with periodontitis in amounts approximately twice those in salivas from normal individuals and that its concentration increases during incubation, it is probably a product of putrefaction regardless of its identity and, therefore, it could easily be δ-aminovaleric acid.

Neither this substance nor volatile amines are known to be natural constituents of the human body. It is probable that they are not present in saliva that is truly fresh, that is, saliva collected without passage through the mouth and adequately stabilized. This raises the question whether the common amino acids found in saliva are natural constituents or the result of bacterial metabolism. A combination of the two is possible and may explain the wide individual variation that has been observed.

Periodontitis and Putrefaction.—The results reported above show that certain differences exist in "fresh" saliva from patients with periodontitis as compared to normal individuals and that these differences are probably the result of putrefaction in the mouth, putrefaction proceeding more rapidly if periodontitis is present. The fact that they remain essentially the same and the amino acid patterns do not differ during incubation may be interpreted to show that whatever is responsible for the differences in "fresh" salivas is not operative during the type of incubation employed. One possible explanation is suggested by the observation that incubation without added casein allows little or no putrefaction to occur. In the mouth free of periodontal disease the only proteins consistently present are those in the saliva which are largely mucoprotein and do not appear to be readily available during aerobic incubation. On the other hand, the periodontal pockets and suppuration characteristic of periodontitis provide a source of protein in the form of destroyed tissue and exudate that is comparable to casein and probably more readily available than salivary protein. In other words, the evidence suggests that extensive putrefaction takes place only when adequate substrate, as is provided in periodontal pockets, is available. This agrees with the often expressed opinion that infection is a secondary factor in periodontitis. It is, of course, not meant to rule out other possible factors, such as aerobic vs. anaerobic conditions and proteolytic enzymes arising from destroyed tissue, which may have as important a role in providing conditions suitable for putrefaction.

SUMMARY

1. The major free amino acids in saliva collected by expectoration are lysine, glycine, alanine, glutamic acid and an uncommon substance which may be δ-aminovaleric acid. At least some of the other common amino acids are present, but in much smaller amounts.

2. Whole hydrolyzed salivas from patients with and without periodontitis do not differ in gross amino acid pattern.

3. Salivas from patients with periodontitis contain larger amounts of unidentified volatile amines and the substance believed to be δ-aminovaleric acid than salivas from normal individuals. It is probable that these are putrefactive products and not natural constituents.

4. These differences remain essentially the same during aerobic incubation in the presence of added casein. Most of the common amino acids appear but their concentrations do not differ between normal and pathologic salivas. In the absence of casein very little putrefaction occurs.

5. The evidence suggests that putrefaction takes place in the mouth only when adequate substrate, in the form of the exudate and destroyed tissue found in the periodontal pockets, is present.

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EFFECT OF CRYSTALLINE CORTISONE ACETATE ON GROWTH OF INTRAOCULAR TRANSPLANTS OF TOOTH GERMS

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GREENE[1] and Browning[2] demonstrated that most embryonic tissues have the capacity for survival and growth when transplanted to the anterior eye chambers of experimental animals. In additional observations on the biologic behavior of transplanted embryonic tissues, Fleming[3] found that the anlagen of teeth also grew and differentiated when transplanted to the anterior eye chambers of experimental animals. During the course of this investigation, it was found that the biologic behavior of transplanted tooth germs varied; some transplants survived and grew for a long period, others rapidly resorbed, and still others grew for a short period and then resorbed. Rapid resorption of some transplants appeared to be the end result of a marked lymphogenic reaction on the part of the host. Woodruff and Woodruff[4] found the anterior eye chamber superior to other bodily locations for receiving transplants, because comparatively fewer lymphocytes attacked transfers to this site. In the present instance, it was felt that a reduction of lymphogenic activity might enhance the growth of tooth germ transplants to the anterior eye chamber and thus increase the number of successful "takes." Crabb and Kelsall[5] found that repeated injections of cortisone caused lymphopenia by reducing the number of circulating lymphocytes. It is the purpose of this communication to present findings relative to the intraocular growth of tooth germs in cortisone-treated guinea pigs.

MATERIALS AND METHODS

Tooth germs were aseptically removed from the jaws of twenty-day guinea pig embryos and were placed in sterile covered Petri dishes where they were kept moist with normal saline until transplanted to the anterior eye chambers of guinea pigs. A representative molar tooth germ from a twenty-day embryo is shown as Fig. 1. Sterility was maintained throughout the transplantation procedure which has been previously described.[3] In the present instance a No. 16 trocar (specially designed) was found suitable for carrying the tissue to the eye. Forty-eight young adult guinea pigs of an average weight of 300 grams were used and of these animals twenty-two were males and twenty-six were females. After receiving the transplants, usually to the right eyes, the animals were divided into two groups of twenty-four each. One group received daily intramuscular injections of approximately 2.5 mg.* of crystalline cortisone acetate (supplied by Merck & Co.) for eight days, while the other group served as controls. Cortisone was discontinued after the eighth day because guinea

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*Due to the tendency of cortisone to froth, and since it was more of a suspension than a solution, precise measurements of injected amounts were impossible.

pigs receiving injections began to lose weight (25 to 30 grams each) and ate poorly. This gave the impression that they were undergoing a toxic reaction from the cortisone therapy. A record was kept of the early biologic activity of the transplants and is shown in Tables I and II. Animals were sacrificed periodically after receiving the transplant from the seventh day up to the sixtieth day. Recovered transplants were formalin-fixed, decalcified, sectioned, and stained with hematoxylin and eosin.

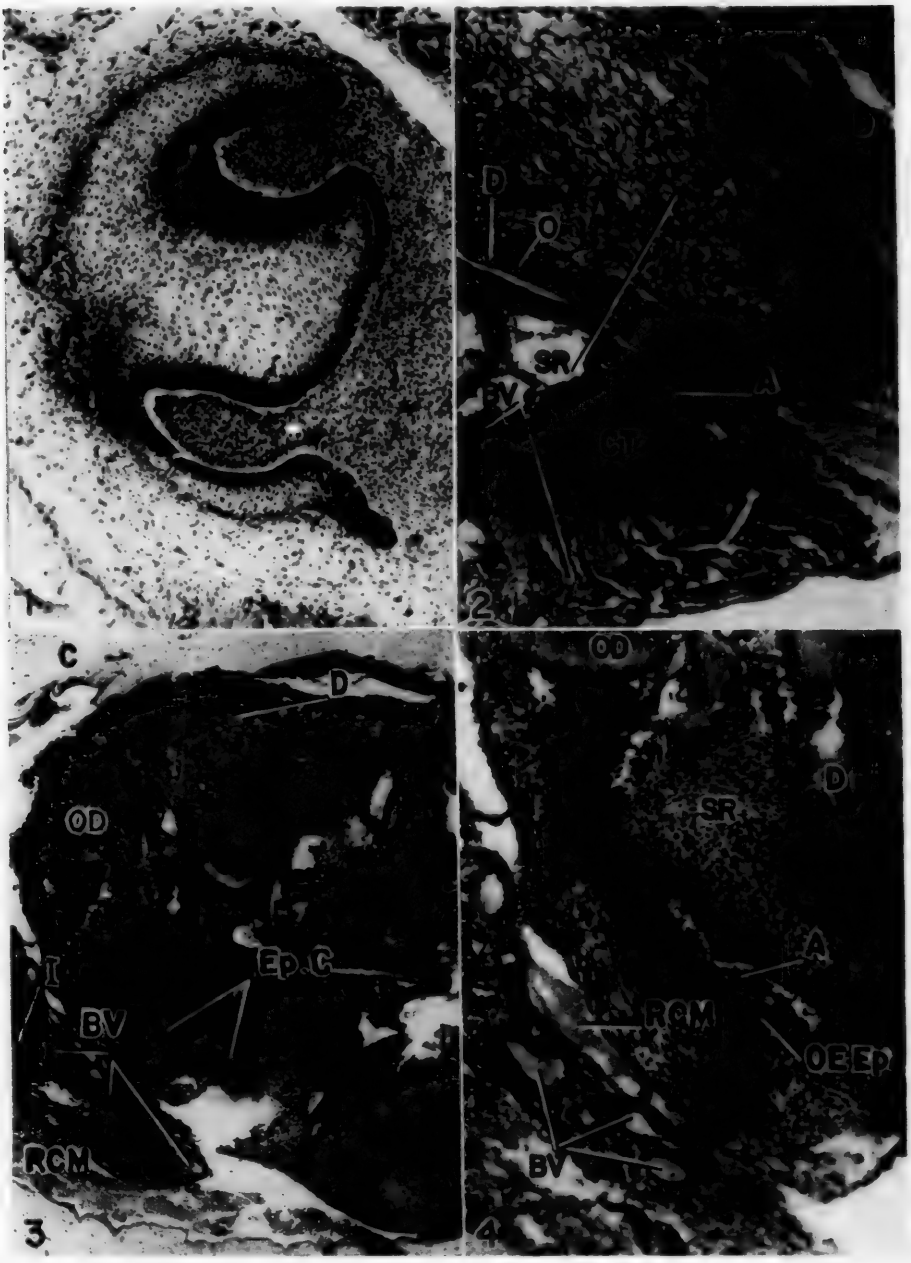
RESULTS

Transplants appeared to vascularize within twenty-four hours in those guinea pigs receiving daily injections of cortisone. In the control group, it was at least four days before some of the transplants assumed a pinkish cast, and generally by the fifth day a more definite evidence of vascularization was apparent. Although the initial growth in the control group was comparatively slow, once these transplants became vascularized growth was so rapid that it soon equaled and subsequently surpassed transfers in animals receiving cortisone. For this reason and also because there were evidences of a toxic effect, cortisone injections were discontinued after the eighth day.

A significant observation was that vascularization of transplants in animals receiving cortisone appeared darker than in the control animals. The reason for this contrast is clarified after studying Figs. 2 and 3. Fig. 2 represents the transplant of a tooth germ after seven days in the eye of a male guinea pig (animal No. 21, Table II). The growth period for the transplant in Fig. 3 was the same as that in Fig. 2; the host also was a male guinea pig (animal No. 6, Table I), but in this case (Fig. 3) the animal received daily injections of 2.5 mg. of cortisone to the thigh muscles. After comparing results in these two cases, it is readily apparent that the growth is much more advanced in the animal that received cortisone, but despite its greater size the transplant seen in Fig. 3 is not attached to the iris. The tooth germ transplant in Fig. 2, although small, is attached to the iris by young connective tissue and the few blood vessels seen in the iris are filled with red blood cells. In Fig. 3, however, the iris is filled with large vascular spaces and the blood vessels observed in it are heavily engorged with red blood cells. In addition, in this latter case, the transplant is almost entirely surrounded by great masses of red blood cells. The large number of vascular spaces surrounding transplants in cortisone-injected animals could account for, in these cases, the darker appearance of the blood. It was also noted that there was an absence of lymphocytes in this instance. It appears (Fig. 2) that the transplant, having survived an initial lymphocytic attack by the host, has just begun to grow. In Fig. 3, the differ-

KEY TO SYMBOLS

A—Ameloblasts
B—Bone
BV—Blood Vessel
Cart—Cartilage
C—Cornea
CT—Connective Tissue
D—Dentin
E—Enamel
Ep C—Epithelial Cells
I—Iris—pigmented layer
KA—Keratinized Ameloblasts
Ly—Lymphocytes
O—Odontoblasts
OEEp—Outer Enamel Epithelium
OD—Osteodentin
RCM—Red Cell Mass
SI—Stratum Intermedium
SR—Stellate Reticulum
T—Transplant
TG—Tooth Germ



Figs. 1 to 4.—

1, Cross section of twenty-day guinea pig molar tooth germ. Original magnification, ×140.

2, Cross section of recovered transplanted tooth germ after seven days in the anterior chamber. Original magnification, ×220.

3, Cross section of transplanted tooth germ recovered from cortisone-treated animal after seven days. Original magnification, ×53.

4, Higher power of Fig. 3 in lower left area. Original magnification, ×165.

(For key to symbols, see page 102)

TABLE I

i/31		ii/1	ii/2	ii/3	ii/4	ii/5	ii/6	ii/7	ii/8	ii/9	
1	♂	Died									
2	♂			Died							
3	♂			Thrown out							
4	♀	+	+	+	+	+	−	−	−	−	
5	♂	+	++	++	++	++	++	+	+	+	
6	♂	+	++	++	++	++	++	++	++		Killed ii/8
7	♀	++	++	+++	+++	+++	++	++	++	++	Killed iv/1
8	♀	+	+?	+	+	−	−	−	−	−	
9	♀	+	++	++	++	++	++	++	++	++	Killed ii/23
10	♀	+	++	+++	+++	+++	++	++	++		Killed ii/8
11	♂	?	−	Thrown out							
12	♂	++	++	++	++	++	++	++	++	++	Killed ii/15
13	♀	+	?	?	Thrown out						
14	♀	+	++	++	++	++	++	++	++	++	Killed ii/23
15	♀	+	++	++	++	++	++	++	++	++	Killed ii/15
16	♀	+	+	?	Thrown out						
17	♀	−	Thrown out								
18	♀	+	++	++	++	++	+	+	+	+	
19	♀	+	+	+	+	+	+	+	+	+	
20	♂	+	+++	+++	+++	+++	+++	+++	+++	++	Killed iv/1
21	♀	+	+	+	+	+	+	+	+	+	
22	♂	+	++	++	++	++	++	++	++	++	Killed iii/3
23	♀	+	+	+	+	+	+	+	+	+	
24	♂	++	+++	+++	+++	+++	+++	+++	++	++	Killed iii/3

2 died
5 were thrown out

First 10 days—vascularization and growth with cortisone.
Animals received 2.5 mg. of cortisone acetate daily for 8 days.

−Regression of transplant.
+Slight growth and vascularization.
++Good growth and vascularization.
+++Excellent growth and vascularization.

TABLE II

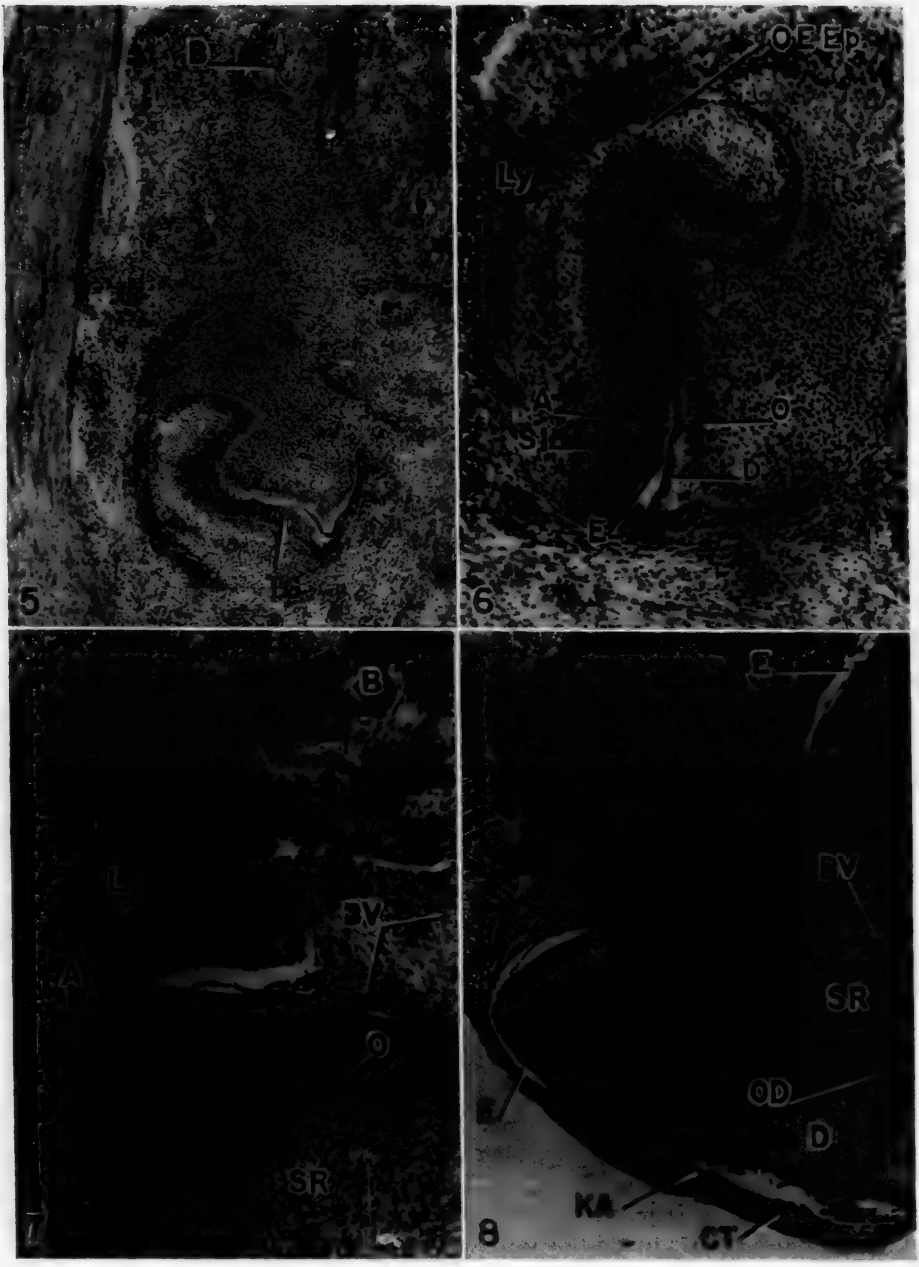
i/31		ii/1	ii/2	ii/3	ii/4	ii/5	ii/6	ii/7	ii/8	ii/9	ii/10	
1	♀	Died										
2	♂	Died										
3	♀					+	++	++	++	++	++ →	
4	♀					+	+	Thrown out				
5	♂					+	+	++	++			Killed ii/8
6	♂				+	+	++	+++	+++	+++	+++ →	Killed iv/1
7	♀					−	−	−	Thrown out			
8	♀					+	++	++	++	++	++ →	Killed iii/3
9	♂					+	+	++	++	++		Killed iii/3
10	♂					+	++	++	++	++	++	Killed ii/23
11	♂					−	−	−	Thrown out			
12	♀				+	+	++	+++	+++	+++	+++ →	Killed ii/15
13	♂				+	+	++	+++	+++	+++	+++ →	Killed ii/23
14	♂					−	−	−	Thrown out			
15	♀					+	++	++	+++	++	++	Killed ii/15
16	♂				+	+	++	+++	+++	+++	+++ →	Killed iv/1
17	♀					+	++	++	++	++	++	Killed iii/3
18	♀					+	+	+	+	+	+	
19	♀					+	+	++	++	++	++	Killed ii/23
20	♀					+	+	+	+	+	+	
21	♂				+	++	++	++	++			Killed ii/8
22	♀					+	+	++	++	++	+ →	
23	♂					+	+	+	++	++	+ →	
24	♀				+	+	++	+++	+++	+++	+++ →	Killed iv/1

First 11 days—vascularization and growth without cortisone.
2 died.
4 were thrown out.

ence in size suggests an enhancement of growth due to serum from the blood and to the lack or absence of a lymphogenic reaction after the transplant was made. Fig. 4 is a higher magnification of Fig. 3 through the lower left corner.

Fig. 5 shows the recovered transplant of a tooth germ after fourteen days in the eye of a male guinea pig (animal No. 12, Table I). While the number of blood vessels and vascular spaces seen in this section indicate greatly increased vascularization, it is decidedly less than that seen in Fig. 3. Fig. 6 is a higher power of Fig. 5. In Fig. 7, which represents the recovered transplant of a tooth germ after twenty-three days in the anterior eye chamber of a female guinea pig (animal No. 14, Table I), development appears more advanced. It should be noted, however, that the ameloblasts seen in the crown portion of this section still appear to be forming the pre-enamel matrix, thus indicating that their activity has been carried over a longer period than usual. The rapidity of tooth development in transplants of tooth germs has been previously observed.[3] Comparatively there seems to be a great advancement in growth from fourteen to twenty-three days. Recovered transplants as represented in Figs. 5 and 7 are from serial sections showing the greatest amount of tooth structure. Calcification appears retarded in Fig. 7. This is the usual observation in recovered transplants from cortisone-treated animals. Lymphocytes are present in both Figs. 5 and 7. The tooth germ shown in Fig. 5 may have grown some, but it now apears to be undergoing resorption. Evidence for this is seen in the focal infiltration of lymphocytes around the transplant. This reaction appears to result from discontinuing the cortisone injections. In Fig. 7, where the development is more advanced, several clumps of lymphocytes are seen near the tooth germ, but there is no general infiltration of round cells or lymphocytes about the transplant.

Fig. 8 shows a part of the recovered transplant of a tooth germ with a well-developed tooth after sixty days in the anterior eye chamber of a male guinea pig (animal No. 6, Table II). It is represented in situ in Fig. 12. Several blood vessels are seen in the stellate reticulum of the pulp and also in the regenerating pigmented layer of the iris. The absence of lymphocytes is significant. In Fig. 9 the growth of the transplanted tooth germ was not as far advanced as that represented in Fig. 8. This transplant was recovered from a male guinea pig (animal No. 20, Table I), after sixty days and is represented grossly in Fig. 8. During the period of cortisone administration it was one of the best appearing transfers but, after cortisone injections were discontinued, this transplant regressed. It is seen grossly in the anterior chamber in Fig. 13 and comparatively it is much smaller and the tooth structure is not as well developed as that in Fig. 8. After cortisone was discontinued, transplants usually became surrounded by lymphocytes and the subsequent activity of these cells appeared to inhibit the growth of the transfer which in many instances seemed to retrogress. Fig. 8 illustrates the transition of dentin into osteodentin, over a sixty-day period; this is from a control animal. In contrast, in Figs. 9 and 11, showing recovered transplants from cortisone-treated animals, there is a significant absence of osteodentin. These latter transplants were also recovered after sixty days.



Figs. 5 to 8.—

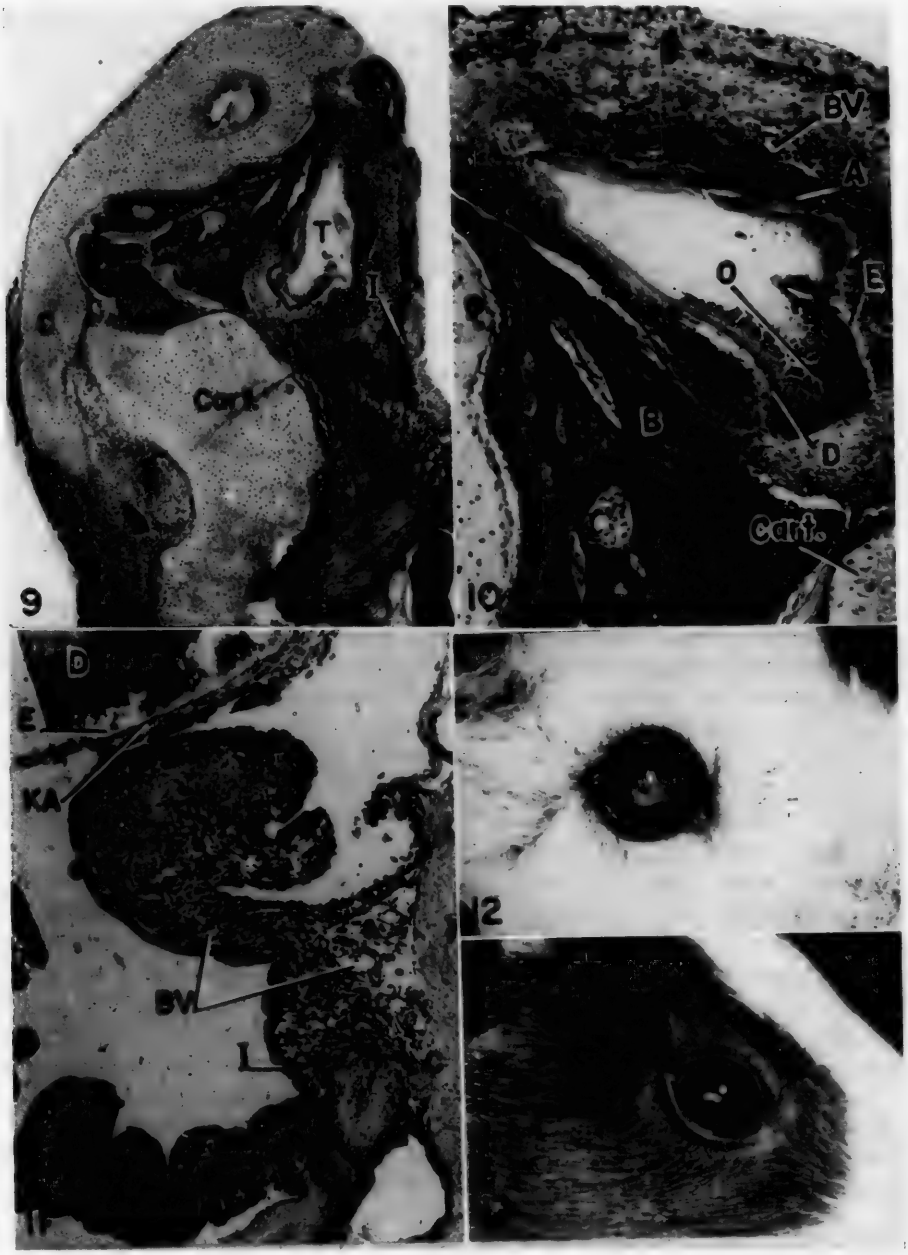
5, Cross section of recovered transplanted tooth germ in cortisone-treated animal after fourteen days. Original magnification, ×60.

6. Higher power of Fig. 5—lower center. Original magnification, ×165.

7, Recovered tooth germ transplant after twenty-two days in cortisone-treated animal. Original magnification, ×165.

8, Recovered tooth germ transplant after sixty days in the anterior chamber. Original magnification, ×165.

(For key to symbols, see page 102)



Figs. 9 to 13.—

9, Recovered tooth germ transplant in cortisone-treated animal after sixty days. Original magnification, ×30.

10, Higher power of Fig. 9 in area of transplant. Original magnification, ×165.

11, Section of recovered transplant of tooth germ after sixty days in cortisone-treated animal. Cut to show mainly the iris. Original magnification, ×165.

12, Transplant in situ after sixty days in untreated animal.

13, Transplant in situ after sixty days in treated animal.

(For key to symbols, see page 102)

Fig. 10 is a higher magnification of the remains of a tooth germ transplant as seen in Fig. 9. Fig. 11 shows a portion of another transplant, in relation to the iris, in a cortisone-treated animal (No. 7, Table I), also after sixty days in the anterior chamber. Despite the high vascularity of the iris adjacent to the transplant, maturation of this transfer is poor in comparison to transplants in untreated animals.

DISCUSSION

The immediate effect of cortisone on intraocular transplants of tooth germs is enhancement of growth. Survival of transplanted tooth germs depends on their relation with the host, for, if they are compatible, rapid development generally takes place.[3] Furthermore, the first several days after transplantation are critical, for during this period transplants of tooth germs usually undergo a lymphocytic attack, which, if successful, means regression and resorption of the transfer. As Woodruff and Woodruff[4] have reported, appearance of lymphocytes in the anterior eye chamber is in response to the presence of the transplant, but lymphocytes do not appear here in as great numbers as at other bodily sites of transplantation. However, the fact remains that lymphocytes do appear at this site and therefore intraocular transplants must be subjected to focal infiltrations of these small round cells. It has been suggested that, through its action upon connective tissue, cortisone inhibits or modifies the functions of the defense mechanisms of the body.[7] Sprague and associates,[8] who reported on the work of many persons, stated that cortisone suppresses white blood cells (lymphocytes and eosinophils) and the activity of lymph glands. Transplants in cortisone-injected animals did not undergo an initial lymphocytic attack and perhaps for this reason transplanted tooth germs became much larger at first than those in animals that did not receive this compound.

Another initial growth-enhancing influence on transplants might have been factors involved which lead to the end results as observed in the present instance, and which seem to be based upon the fundamental alteration of hormones or hormone balance in the host animals under the influence of cortisone.

It has been reported by Finch, Crockett, Ross, and Bayles[9] that using cortisone for treating patients with rheumatoid arthritis caused an increase in the total red cell mass of all anemic patients. The findings of the above clinical investigators could be applied to the present experiment and could explain the tremendous increase in red cell masses as seen in vascular spaces around the transplants. Furthermore the increase and dilation of blood vessels in the irises of cortisone-treated animals indicate a possible change in the endothelial systems of these hosts due to the activity of this adrenal steroid.

The fact that transplants in cortisone-treated animals did not readily become attached to the iris is also of great significance and it may be partly explained by recent investigations of Barber and Nothacker.[10] They found a delayed wound healing, which was occasioned by the belated appearance of fibroblasts and the prolonged immaturity of collagen, in cortsisone-treated animals. After connective tissue attachments were completed by transplants in animals receiving no cortisone, growth and differentiation (in these cases)

were greatly accelerated and soon surpassed the growths in cortisone-treated animals. Transplants in cortisone-injected animals underwent a lymphocytic attack immediately following the cessation of injections. For this reason many transfers were soon resorbed and those that survived were small in comparison to the ones in untreated animals.

CONCLUSION AND SUMMARY

1. Transplants of tooth germs in cortisone-treated animals appeared to vascularize immediately, and growth of these transplants was at first rapid.

2. The cellular response to transplants was depressed in cortisone-treated animals.

3. Growth of tooth germs in untreated animals was very slow at first, but after vascularization took place, quickly surpassed growths in cortisone-treated animals.

4. Many transplants resorbed in cortisone-treated animals when the injections were stopped.

5. Growth was superior in uninjected animals.

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PHYSIOLOGIC STUDIES ON THE INNERVATION OF TOOTH

Electrical Impulses From the Pulp Nerve Fiber

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GAKURO HARA, AND MINORU MASUDA

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IT IS a matter of common observation and clinical experience that the tooth has a rich sensory reaction. Hypersensitive dentin is frequently experienced during dental treatment. Therefore investigation of this problem should be one of the most important subjects in the dental area.

The earliest workers considered that the apparent response of dentin to stimuli was due to mechanical impulses transmitted to pulp. Clinical researches by Duval (1833)[1] and Bell (1835)[2] showed that the dentin itself is acutely sensitive to pain and this has been the widely accepted view up to the present time.

On the other hand, it had been found that as the fibers of dental nerves proceed up through the pulp they progressively lose their myelin sheaths. To explain the sensitivity of the dentinoenamel junction, some investigators (Huber,[3] Noyes,[4] Bödecker,[5] Hanazawa,[6] Terasaka[7]) considered that the nerve may terminate in the region of the odontoblasts in special endings, and other investigators (Mummery,[8] Adrion,[9] Toyoda,[10] Sealey,[11] Allen,[12] Stewart[13]) claimed that the nerve fiber may continue for varying distances into the dentin as fine fibers. Demonstration of the existence of nerve fibers in the dentin should clarify the problem. Another explanation was suggested by Hopewell-Smith,[14] Bödecker,[15] and Cabrini,[16] that the nerve fibers may end in the region of odontoblasts and that these cells may be sensory end organs. Therefore, if stimuli were given at the dentinoenamel junction, the odontoblasts would be stimulated and they would conduct the excitation to the pulp nerve which terminates at the region of the odontoblasts.

Most studies of this subject have been discussed only from the standpoint of histology, and little attention has been given to the physiologic studies except by Pfaffmann.[17, 18] He used electrophysiologic methods to register the action current in the dental nerves of the cat. His work was done on the afferent impulses caused by pressure, vibration, and noxious stimulation to the teeth. He did not demonstrate the cause of the sensibility of the dentinoenamel junction. In this report, therefore, the mechanism of the conduction of the dental sensory nerve impulses was investigated by recording the action potential induced from microelectrodes inserted in dental nerve trunks.

METHOD

The recording apparatus consisted of a resistance capacity coupled amplifier and a cathode-ray oscillograph arranged for visual scanning as well as photography. An induced microelectrode is shown in Fig. 1. Its diameter was

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20 to 30 microns, and into its glass tube a fine silver wire, 7 to 10 microns in diameter and coated with silver chloride, was inserted. As the subjects, the canine and other teeth of the lower jaw of dogs were used.

Experimental procedure was as follows: The dog's head and lower jaw were rigidly held on the operating table by a specially made clamp. The nerves supplying the canine or other teeth were located in dogs anesthetized by Evipan

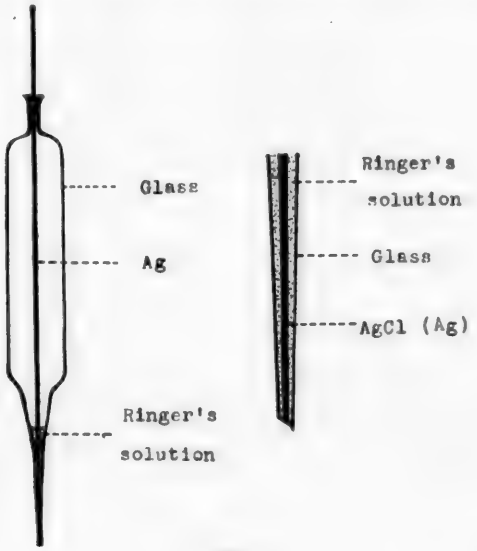

Fig. 1.

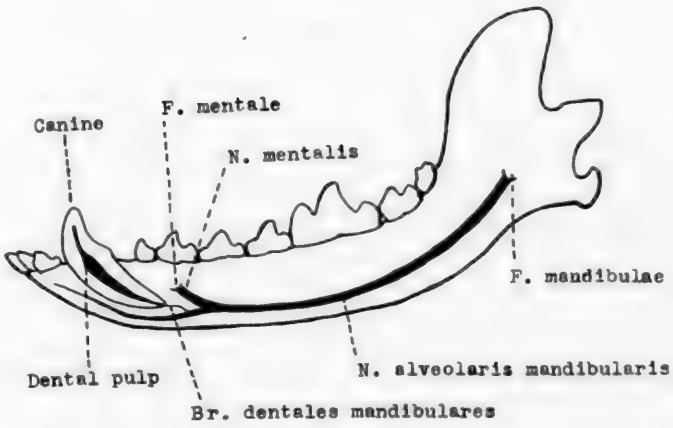

Fig. 2.

natrium. The mandibular bone was exposed by removing the skin. The dental branches of the mandibular nerve, as they pass through the alveolar canal of the mandible, were exposed (Fig. 2), and precaution was taken to avoid wasteful bleeding.

The animal was placed in a heated chamber and the temperature maintained at about 30° C. to keep the nerve in good condition. After confirming the existence of the dental nerve (dentales mandibulares) at the root apex, the microelectrode was inserted into that nerve. The other indifferent electrode was placed on the bone tissue near this electrode and they were connected to the amplifier. In all these experiments, the operator must put on rubber gloves to prevent the induction of alternating current to the amplifier.

The tapping of a knife handle, hot air jet, cold water, and the vibratory stimulus of a dental bur or point were used. Occasionally electrical inductive current was also used. In these experiments, generally, the threshold of the stimuli was not measured, as the purpose of this report was to clarify the source of the sensitivity of the dentinoenamel junction. When electrical responses arose from the stimuli, the examined teeth were prepared by grinding, and the stimulated loci were inspected in these preparations.

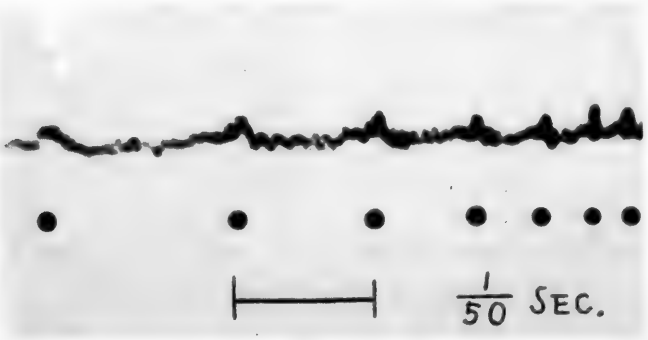

Fig. 3.

RESULTS

When the tapping of the knife handle or electrical stimuli are given to the intact tooth, no electrical discharge of nerves is elicited nor does electrical discharge result when the neighboring intact teeth or gingivae are stimulated. This result differs from that obtained by Pfaffmann. When enamel is stimulated by the dental bur or point, the same reactions are obtained (Fig. 3). If the mechanical stimuli approached close to the dentinoenamel junction (Fig. 4), the electrical sign of the nerve suddenly appeared. One of these reactions is shown in Fig. 5. Removal of the stimuli results in immediate cessation of the afferent impulses. A jet of hot air produces a volley of discharges (Fig. 6), but cold water and electrical stimuli are not as effective. On these points Pfaffmann pointed out the same results. On using the electrical stimulus, under the same conditions, the nerve responses are concealed by an electrical artefact affecting the amplifier, so that the irregularity of the base line of oscillograph makes it difficult to recognize the response.

When the dentin was stimulated, the action current could not be proved and occasionally a few spikes were shown during a long interval in the absence of

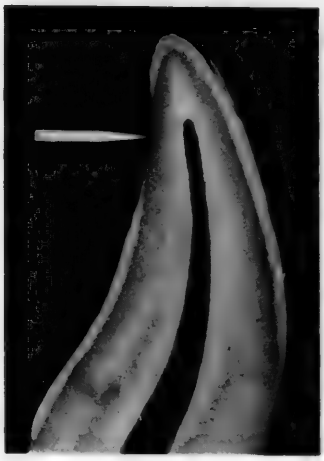

Fig. 4.

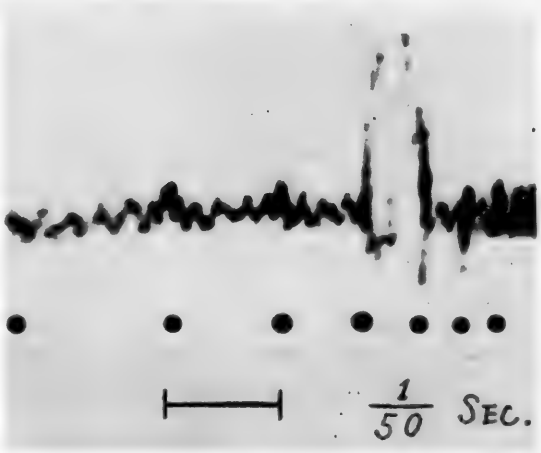

Fig. 5.

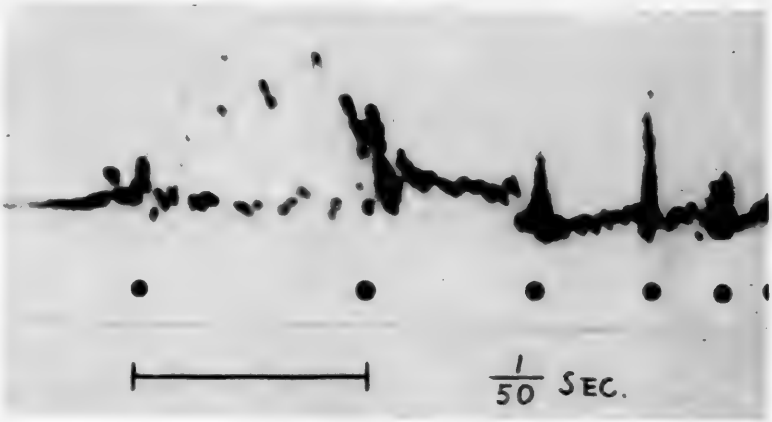

Fig. 6.

stimulus. These action currents are named the "spontaneous discharges" (Fig. 7). The results are worthy of note to explain nerve termination. When the stimulus extended to the pulp and reached the area of predentin or odontoblasts, the spikes could be found again. When the mechanical stimuli reached the pulp cavity, however, the action current could not be demonstrated. Electrical stimuli were used at the same time, but at this time the excitation of the nerve fiber could not be found for reasons just given.

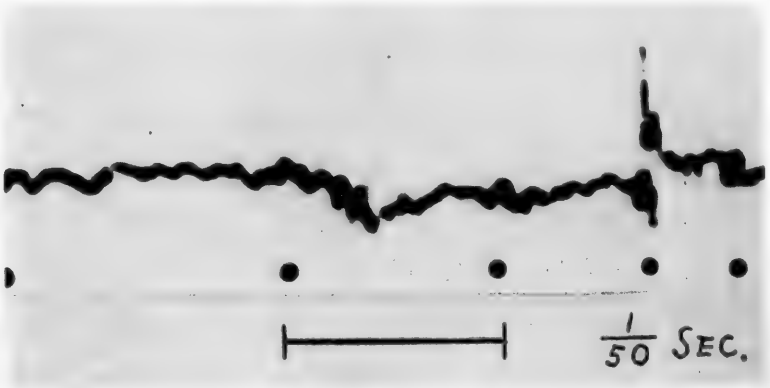

Fig. 7.

DISCUSSION

Tomita,[19] and Woodsbury and others[20] pointed out the possibility of taking photographs of a single nerve fiber's action current from the nerve trunk by using a microelectrode of 1 to 5 microns diameter. However, in our studies the potentials seem to be the mixture of several fibers. Perhaps the reason may be that the electrode's diameters are 20 to 30 microns, and they catch the action current from a few fibers near the electrode. Accordingly, the nearest nerve fiber to this electrode shows the highest potential, and other fibers, according to their distance to the electrode, show smaller heights.

The finding that nerve impulses are activated by stimulation to the dentinoenamel junction, and by the excitation of the area of predentin or odontoblasts, is in keeping with clinical observation in man.

Pfaffmann's findings showing that the pressure threshold of teeth before and after the removal of the pulp is little changed and that the rich tactile response may be obtained after the destruction of the apical nerves agree with the histologic finding that the majority of the nerves to the periodontal membrane come from the alveolus itself. Different results were found in our experiments, the response not being demonstrated by tapping the teeth or electrical stimuli to intact teeth. The difference between Pfaffmann's data and ours on touching the teeth or gingiva may be due to the difference of method. The differences in our experimental conditions and his suggest the innervation of the dental nerve to the pulp and to the periodontal membrane.

The fact that in our study, electrical responses are not demonstrated upon stimulation to the dentin is of interest in explaining the sensation at dentinoenamel junction. On the other hand, Pfaffmann showed in one case that merely by exposing the dentin a discharge of moderately fast impulses which could be diminished by covering the exposed part with absorbent cotton soaked in warm Ringer's solution was elicited. Furthermore, Pfaffmann indicated that hot water was more effective than ice water, a finding confirmed by us. The spontaneous discharges on the stimulation of dentinal tubules may be explained by the Pfaffmann theory. For these reasons obliterated dentin tubules may be considered either as a nonexcitable region or as a region that has no nerve fibers.

Many investigators have demonstrated that nerves exist in the region of predentin or odontoblasts and that ends of some nerve fibers terminate at the odontoblast cells and those of others at the predentinal tubules. Hence, nerve impulses would be expected to appear as the result of stimuli to this area, and our results quite agree with this presumption.

From these results, the theory that fine fibers may continue into the dentin cannot be accepted as the true explanation of the cause of hypersensitive dentin. The neurofibril is undoubtedly intimately associated with the odontoblasts. The results of Mummery and others may be due to technical failure. The hypothesis of Hopewell-Smith and others, that the odontoblasts are sensory end organs, cannot be yet regarded as the correct explanation according to our present experimental results. The appearance of spikes seems to indicate a time delay from the stimulus, although in this experiment it was not measured. Perhaps this may support the theory of Hopewell-Smith from a physiologic point of view, a possibility under investigation.

SUMMARY

Physiologic investigations were made to explain the etiology of hypersensitive dentin. The results obtained were as follows:

1. Electrical afferent impulses from the mandibular nerve were evident when dentinoenamel junction, predentin, or odontoblasts were stimulated.

2. While rubbing dentin, the impulses could not be found, but, occasionally, spontaneous discharge was elicited.

3. These results suggest that the nerve fibers do not terminate in the dentinal tubules.

4. A hot air jet was more effective than cold water and electrical stimulus in causing the excitation.

We desire sincerely to acknowledge our indebtness to Dr. T. Okumura, Dean of Tokyo Dental College, for his constant encouragement and financial aid.

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THE EFFECT OF X-RAY IRRADIATION ON THE RAT INCISOR

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THE first extensive survey[1] of the radiologic literature by Shields Warren (1942-1943) and subsequent experimental[2-7] and clinical[8-14] reports, revealed that the effects of radiation on oral tissues have not been thoroughly investigated. As early as 1905, Tribondeau and Récamier[15] noted radiation-induced dental changes after irradiating the right side of a kitten's face with six ten-minute roentgen-ray exposures, administered three times a week at 10 cm. distance. They observed underdevelopment of the skull on the irradiated side with retardation in the development of the teeth more marked than that in the development of the bones of the face. Approximately two decades later Leist[16] (1927), Herold[17] (1931), and Smith[18, 19] (1931, 1938) reported similar but more detailed observations on irradiated young rats and dogs. They postulated that gamma radiation had a selective effect on the odontoblasts with a temporary disturbance in dentin formation, delayed eruption of the teeth, and diminished growth of the roots. No effect was noted in the enamel as a result of radiation.

Although recent experimental investigations[3, 6] have contributed much information regarding the histopathology of irradiation on oral tissues of heavily irradiated mice[3] and swine,[6] the following report discloses radiologic effects in incisal rat enamel of normal, living animals. These changes which had not been previously reported are seen microscopically in unstained ground dental sections.

MATERIALS AND METHODS

The animals for this study were obtained from Dr. Robert Boche's experiment to determine the effect of age and sex on mortality from whole-body x-ray irradiation. Three hundred albino rats of equal sex distribution were divided into five groups of 30 weanlings (at 55 days of age) and 30 adults (at 200 days old) which were exposed to single whole-body exposures of 0, 375, 500, 625, and 750 roentgens (r) administered under constant conditions, viz., 250 kv., 15 Ma., Al parabolic filter, and ½ mm. Cu, H.V.L. 2.1 mm. Cu for 44 minutes. The target skin distance (T.S.D.) and roentgens per minute for each group were: (I) Control T.S.D., 0; r per min., 0; (II) 375r, 35.5 inches T.S.D.; 8.5r per min.; (III) 500r, 30.5 inches T.S.D.; 11.4r per min.; (IV) 625r, 27.5 inches T.S.D.; 14.2r per min., and (V) 750r, 25 inches T.S.D.; 17r per min. Oral tissues of animals dying before termination of the experimental period (30 days) were examined grossly under a dissecting microscope (X30), but not histologically. Thirty days after radiation exposure, the survivors

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were sacrificed and the oral tissues were examined grossly (X14 and X30). Eight living animals per group were randomly selected for microscopic study by preparing ground and decalcified sections from respective halves of the maxilla and mandible of each rat. Ground sections were prepared under running water to about 40 micra thickness. Tissues for decalcification were fixed* in Zenker's formalin solution; decalcified† in lactic-nitric acid mixture, and embedded in celloidin. All decalcified sections were stained with hematoxylin and eosin.

RESULTS

Mortality data disclosed a slightly greater incidence among males with no apparent age difference: (I) Controls, 0; (II) 375r, 1 death (adult female died from otitis media); (III) 500r, 6 deaths (3 males); (IV) 625r, 22 deaths (16 males), and (V) 750r, 50 deaths (28 males).

Generally speaking, gross examination under the dissecting microscope revealed, with increasing exposures, increasing periodental disturbances characterized by edematous and bleeding gingivae, occasional nasopharyngeal and oral mucosal petechiae, and ulcers. Morphologically the incisors and molars appeared normal; however, a delayed eruption and questionable enamel hypoplasia were believed to have been observed in the incisors of Groups IV (625r) and V (750r), while the molars were unaffected.

Microscopically there was evidence of a temporary retardation or cessation of both enamel and dentin formation in the incisors with no noticeable disturbance in calcification and apparently normal recovery.

The characteristic enamel dip typical in ground sections for Group II (375r) animals is seen at 10 magnification in Fig. 1. A higher magnification (100X) of the maxillary incisal defect is seen in Fig. 2. The incisal pigment and the labial and lingual dentin appear to be undisturbed, while the enamel structure before (*A*), during (*B*), and after (*C*) radiation exposure is clearly delineated. The hypoplastic enamel findings were consistently seen in unstained ground sections, whereas no significant dentinal changes were observed histologically in either ground or decalcified microsections. At 375r the incisal enamel was affected more than dentin and was characterized by smaller irregular, amorphic and discolored enamel rods with dark inclusion bodies in the interprismatic spaces. Since hematologically there were no significant changes at 375r (Group II), this finding suggests a marked ameloblastic radiosensitivity.

As radiation increased, both amelogenesis and dentinogenesis were progressively inhibited, and the degree of hypoplasia increased respectively. Typical incisal changes for albino rats exposed to 500r (Group III) are seen in Figs. 3 and 4 at 100 and 430 magnification. The pre-exposed enamel (*A*) and dentin, the hypoplastic enamel (*B*) and dentin (circled), and the postradiation recovery (*C*) are easily visible. Dentinal growth was definitely inhibited, but the enamel had more disturbance and appeared completely amorphic (Fig. 4). There was no apparent effect on calcification. Leist[16] and Smith[18, 19] proposed

*Fixation: Zenker's solution 90 per cent; formalin 10 per cent by volume.

†Decalcifying solution: Concentrated lactic acid 7 per cent; concentrated nitric acid 2 per cent; and distilled water 91 per cent by volume.

Fig. 1.

Fig. 2.

Fig. 1.—Ground, unstained maxillary (left) and mandibular (right) incisors of weanling male rat (10×) showing characteristic enamel hypoplasia for Group I animals (375r). Note normal uniform incisal pigment layer with little or no effect on labial or lingual dentin.

Fig. 2.—Higher magnification of maxillary incisor (100×) of previous slide (Fig. 1). Note enamel rod structure before exposure (*A*); irregular, disturbed, or hypoplastic enamel due to radiation (*B*), and the excellent recovery or regrowth potential (*C*). The underlying dentin appears unaffected.

Fig. 3.

Fig. 4.

Fig. 3.—Ground section 100×; adult male incisor exposed to 500r. Pigment layer appears undisturbed. Both enamel (*B*) and dentinal (circled) development were diminished with no apparent effect on calcification of dentin. Postradiation development of enamel (*C*) and dentin appear normal, even though histologically ameloblastic interference (Fig. 4) appears to be greater than odontoblastic activity.

Fig. 4.—Higher magnification (×430) of Fig. 3. Note completely amorphic enamel rod structure with dark inclusion bodies on interprismatic substance.

Fig. 5.

Fig. 6.

Fig. 5.—Ground section of maxillary incisor 10×; weanling female exposed to 625r. Note complete inhibition of enamel and dentin formation with excellent recovery at formative end. Position of incisal hypoplasia suggests delayed eruption; same animal was killed thirty days after exposure and the incisors regenerated completely in about forty-five days.

Fig. 6.—Decalcified section (10×) of adult maxillary incisor exposed to 625r. Note the inhibited growth of dentin with normal regrowth potential. Effect appears to be specifically in the laying down of dentinal matrix and odontoblastic activity, not in calcification. The position of the defect again suggests delayed eruption. No satisfactory explanation can be given for failure of disturbance of the lingual dentin.

that the disturbance was in the calcification of dentin, while Bruce and Stafne[13] reported a premature calcification of permanent teeth as seen in roentgenograms. These data suggest an ameloblastic as well as an odontoblastic inhibition with no apparent histologic effect on calcification. The disturbance probably is due to an interference or inhibition in the laying down of enamel and dentinal matrices.

In Figs. 5, 6, and 7 are seen changes for adult and weanling maxillary incisors receiving 625r (Group IV). Complete inhibition of enamel and dentin formation is seen in the ground section (Fig. 5) at 10 magnification with excellent receovery at the formative end. In decalcified sections (Figs. 6 and 7) a temporary retardation or cessation of dentinogenesis is manifested which is

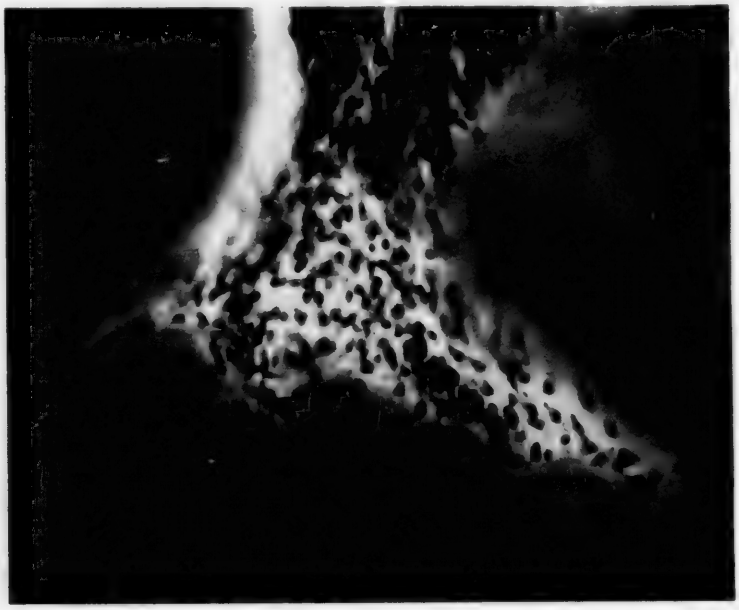

Fig. 7.—Higher magnification of Fig. 6 showing marked disorganization in odontoblastic layer with degenerated odontoblasts enclosed in a dentinoid or osteodentinoid material as described by Leist (1926), Smith (1931), and Burstone (1950).

characterized by a marked disorganization of the odontoblastic layer with degenerated, shrunken odontoblasts enclosed in dentinoid or osteodentinoid material as described by Leist,[16] Smith,[18, 19] and Burstone.[3] In comparison to the control incisors, the postradiation incisal development seemed smaller, but this finding was somewhat inconclusive due to the small number of animals (eight per group) examined microscopically. However, the position of the incisal hypoplasia due to radiation definitely indicates a retardation in eruption since the animals were sacrified thirty days after x-ray exposure, and rat incisors regenerate completely in about forty-five days.[20, 21] If eruption were not retarded, the radiologic defects would be located in the incisal third or the

erupted portion. Also with greater exposures the incisal hypoplasias are located more posteriorly in the unerupted part; for example, Figs. 5 and 6, suggesting greater retardation in eruption with increasing radiation.

Radiologic effects in the molars were more pronounced in the radicular areas, particularly the third molars of the weanling rats. These effects were characterized by dwarfed or diminished root formation, by larger radicular and coronal pulps, and by decreased deposition of secondary dentin and cementum. Histologically, the adult rat molars exhibited little or no significant morphologic change except in the peridental tissues and oral mucosa.

Typical radiologic reactions of the oral epithelium were seen infrequently in the gingivae and oral mucosa (edematous, bleeding gingivae, hemorrhagic petechiae, and ulceration). The severity and frequency of the lesions varied with the degree of radiation exposure and with the health of the animals. These findings were not as consistent as the dental observations.

DISCUSSION

The continuously developing rat incisor is a valuable biologic indicator of the metabolic status of the animal, for developing enamel and dentin record a histopathologic pattern of factors which alter calcification of tooth structure. Unlike transitory changes in bone, the histologic pattern cannot be altered since calcium is usually not withdrawn from teeth and therefore furnishes a permanent record of changes in mineral metabolism over a period of time. Since rat incisors are extremely actively metabolizing tissues, erupting about 2.1 to 2.2 mm. per week for the uppers and 3.1 to 3.2 mm. per week for the lowers,[20, 21] the finding of radiation-induced changes in developing enamel is not unexpected because the ameloblast is a highly specialized cell of epithelial origin, and, therefore, high in the list of radiosensitive cells.[23] The possible mechanism for the development of enamel hypoplasia from radiation has been described by English and Tullis.[6]

Most radiologists believe that 98 per cent of albino rats surviving thirty days after radiation exposure will continue to live, and are normal as far as all chemical and physical tests indicate. There may be some disagreement on this point, however, since a few animals may die within sixty days and the decrease in life span is unknown.[22] It is interesting to note that at whole-body exposures of 375r (Group II), there were no significant hematologic findings, yet the incisal enamel changes were distinct and consistent in all animals regardless of age and sex. With increasing radiation the severity and degree of radiologic effects in the teeth and oral mucosa increased respectively. The possible effects of chronic irradiation on the rat dentition[24] will be reported in a subsequent report.

Earlier investigators[16, 17, 18, 19] reported no disturbance in any of the hard structures except the odontoblasts and the dentin. The effect was only temporary and only the area undergoing development at the time of exposure was affected. They declared that the disturbance was in the calcification of the dentin. In general, these observations were confirmed. In addition, a definite inhibition of amelogenesis, which seemed to be more radiosensitive

than dentinogenesis, was observed. Calcification appeared normal, and the radiologic effect is probably in the laying down of the enamel and dentinal matrices. Ameloblastic disturbance has been reported by subsequent investigators in rats[3] and swine.[6] As for calcification, Bruce and Stafne reported a premature calcification of permanent teeth in human beings by means of roentgenograms.[13]

It is needless to mention the importance of recognizing radiologic effects in oral tissues with the increasing use of atomic energy for medical, industrial, and military purposes. Radiation-induced dental changes may be manifested in the developing tooth buds of atomic warfare survivors who have received undue radiation, as well as cancerous patients undergoing prolonged radiotherapy in the cervicofacial regions without undue dental protection. Dwarfing of the teeth, anodontia, delayed eruption, premature calcification, osteoradionecrosis, noninflammatory pulpitis, xerostomia, rampant caries, as well as edematous, bleeding gingivae, hemorrhagic petechiae, and ulceration may be common lesions.

CONCLUSION

The oral tissues of five groups of 30 weanlings (55 days) and 30 adult (200 days) albino rats of equal sex distribution receiving single, whole-body x-ray exposures of 0, 375, 500, 625 and 750 roentgens under constant conditions were examined grossly under a dissecting microscope. Eight living animals per group were studied microscopically with ground and decalcified sections prepared from respective halves of the maxilla and mandible.

Gross examination revealed increasing periodontal pathosis characterized by edematous bleeding gingivae, hemorrhagic petechiae, and some ulceration of the oral mucosa and nasopharynx. The soft tissue lesions varied with exposure and the state of health of the animal. Morphologically the incisors and molars appeared normal.

Microscopically there was a temporary retardation or cessation of both amelogenesis and dentinogenesis, and delayed eruption of the incisors with no noticeable effect in calcification and apparently normal recovery. Radiologic effects in the molar dentition were limited to the third molars of weanling rats which manifested larger radicular and coronal pulps, shorter roots, and decreased deposition of secondary dentin and cementum. At 375r there were no significant hematologic findings, yet the incisal enamel was inhibited more than the dentin, suggesting a marked ameloblastic radiosensitivity.

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A CYTOLOGIC STUDY OF SALIVARY GLANDS OF THE MOUSE TONGUE

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THE salivary glands of the tongue of the mouse are located near the base of the tongue and are surrounded by striated muscle. Mucous glands which are arranged in a spherical mass are found in the area of the vallate papillae. Adjacent and medial to the mucous glands are the serous glands of von Ebner. The latter consist of both individual alveoli and groups of alveoli arranged around ducts.[1, 2]

In this study certain histochemical methods were employed in order to demonstrate and to distinguish between the intracellular precursors of mucous and serous gland secretions. Furthermore, the methods used may also indicate differences in the submicroscopic state of organization of these substances. The methods, in conjunction with fixation by freezing and drying, may elucidate certain cytochemical aspects of normal salivary gland tissue which have heretofore been obscured by the labile nature of these tissues when subjected to conventional aqueous fixatives.

METHODS

The tongues of fifteen young adult C57 mice were removed and fixed by freezing and drying.[3] The specimens were frozen in isopentane chilled by liquid nitrogen (approximately –15° C.), and then dehydrated in a vacuum (approximately –32° C.). Tissues were infiltrated and embedded in paraffin (melting point 62° C.) in a vacuum, and sectioned serially at 6 microns. The sections were then dry mounted on albuminized slides and deparaffinized with xylol or petroleum ether. The latter was used to deparaffinize sections intended for specific solubility and enzyme tests. Some sections were denatured overnight in ethanol prior to staining, while others were denatured only following treatment with certain reagents.

For purposes of orientation undenatured sections were stained with hematoxylin and also mucicarmine. The latter is known to give a positive staining reaction for mucus-containing alveoli, but fails to stain serous alveoli.[4] These older observations on the staining reaction of salivary glands with mucicarmine were made on tissues fixed in aqueous fixatives. Since loss of water-soluble components in addition to chemical charges caused by fixation may have occurred, it was felt that the previous observations on the staining reaction with mucicarmine should be confirmed using frozen-dried tissues.

Undenatured sections were stained with an aqueous solution of toluidin blue (1-1000), cover slipped, and observed as wet preparations[5] in order to study metachromatic staining reactions, since metachromasia of mucous salivary glands has been observed in tissues fixed in aqueous fixatives.[4] It is believed

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that the interaction of cellular components with dyestuffs to produce a metachromatic staining reaction may be related to the state of organization or aggregation of the intracellular substances.[6]

A modification of the Unna-Pappenheim methyl green-pyronin* method was also used to stain a series of undenatured sections. Recent work has indicated that the selective affinity of certain cellular components for either methyl green or pyronin may be related to their state of aggregation or polymerization.[7] Methyl green-pyronin was prepared according to the method described by Cowdry[8] with the following exceptions: The methyl green was extracted repeatedly with chloroform in order to remove crystal violet which is present in appreciable quantities, and the solutions were prepared with a pH 4.1 acetate buffer which has been shown to result in a greater stability of methyl green to light.[7]

A group of sections was stained for carbohydrate-protein complexes (glycoproteins) and glycogen by use of the periodic acid-leukofuchsin method.[9, 10, 11] This histochemical method is particularly applicable for the study of salivary gland components, since their secretory products have been chemically identified as containing glycoproteins.[12, 13, 14] Furthermore, this method in conjunction with certain enzyme and solubility tests is believed to give an indication of the state of aggregation of glycoproteins.[15]

The periodic acid-leukofuchsin method employs an oxidizing agent, periodic acid, to oxidize certain hydroxyl groups of the carbohydrate portion of the glycoprotein to aldehyde groups. These aldehyde groups react with colorless leukofuchsin to form a dye compound which stains pale pink to red-violet. In addition to staining glycoproteins and glycogen, the periodic acid-leukofuchsin method also stains monosaccharides, free aldehydes, and lipoprotein compounds. These substances, if present, may be eliminated in tissue sections by use of appropriate solvents prior to staining.[16] In the tissues studied, simple sugars, free aldehydes, and lipoproteins apparently were not present to a degree which significantly affected the staining reaction. To distinguish between glycoproteins and glycogen, both of which stain with this procedure, a group of denatured sections was treated with amylase† at 37° C. (alpha and beta amylase 1 mg. per cubic centimeter, pH 6 phosphate buffer, M/15) for 30 minutes prior to staining in order to remove the glycogen. Control slides were incubated in the buffer solution alone.

A series of undenatured sections was treated prior to staining with the enzymes collagenase‡ (1 mg. per cubic centimeter in Palitzsch's borate buffer,[17] for one hour at 37° C.), and trypsin§ ($\frac{1}{10}$ mg. per cubic centimeter in pH 7 phosphate buffer, M/15 for five and fifteen minutes at 37° C.). Control slides were incubated in the buffer alone. Both collagenase and trypsin are enzymes which are known to alter some proteins including protein-carbohydrate substances.

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*Methyl green, National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y., C.I. No. 685; pyronin B, National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y., C.I. No. 741.

†Nutritional Biochemicals, Cleveland, Ohio.

‡Type A collagenase furnished by the Wellcome Research Laboratories, Beckenham, England.

§The Armour Laboratories, Chicago, Ill.

Fig. 1.

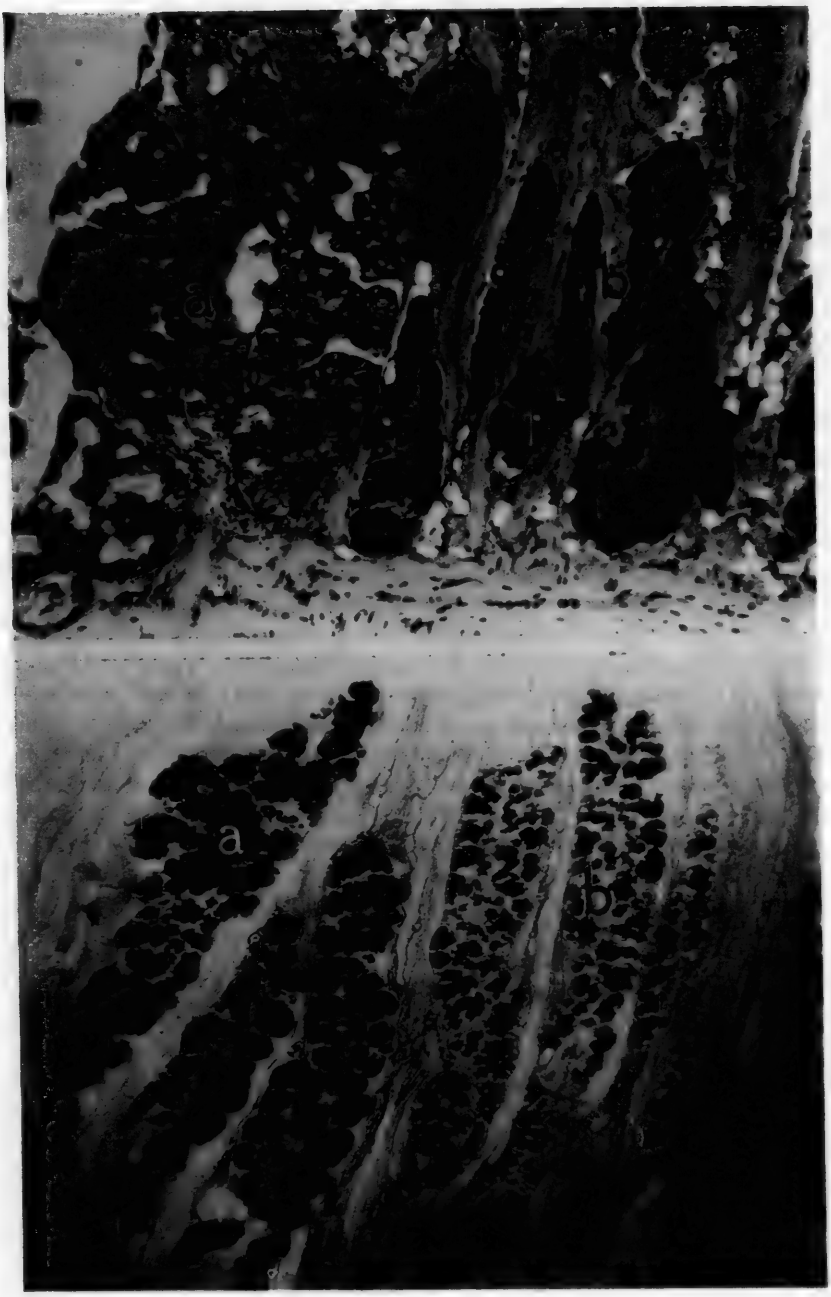

Fig. 2.

Fig. 1.—Mucous glands (*a*) and serous glands (*b*) of tongue stained with hematoxylin and eosin (orig. mag. ×140.)

Fig. 2.—Mucous glands (*a*) and serous glands (*b*) of tongue stained according to the periodic acid-leukofuchsin method (orig. mag. ×140.)

Fig. 3.

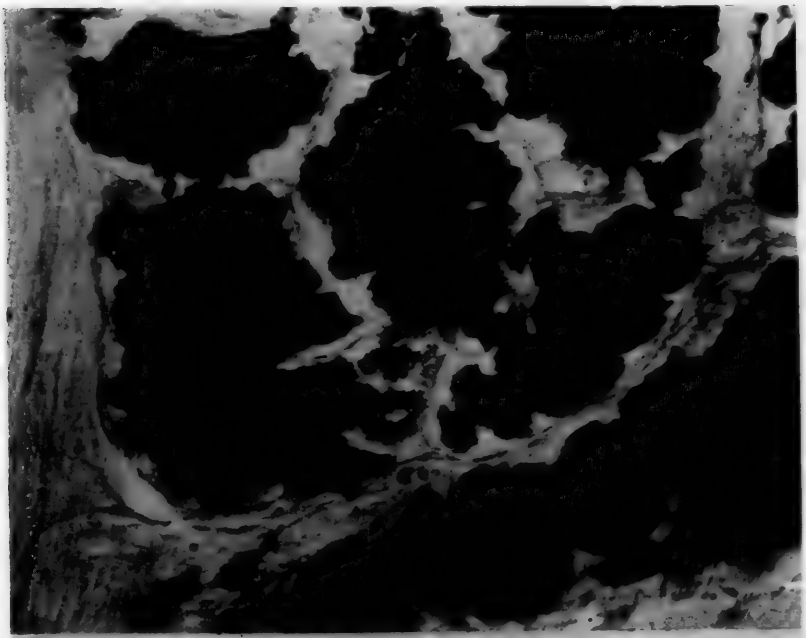

Fig. 4.

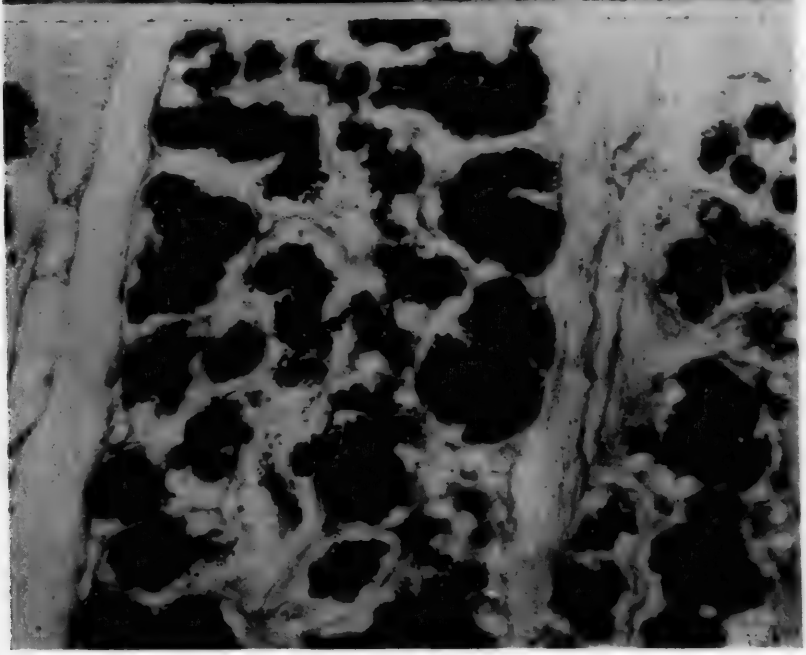

Fig. 3.—Higher magnification of mucous alveoli. Periodic acid-leukofuchsin staining method (orig. mag. ×640).

Fig. 4.—Higher magnification of serous alveoli, showing glycoprotein granules (orig. mag. ×640). (Photograph taken using a Wratten filter No. 58 in order to accentuate contrast.)

OBSERVATIONS

Hematoxylin and Eosin and Mucicarmine—The salivary glands of frozen dried material stains with hematoxylin and eosin similarly to that of formalin-fixed tissue (Fig. 1). Mucicarmine stains only the mucous alveoli, as has been observed with mucous gland tissue fixed in conventional aqueous fixatives.

Toluidin Blue—Wet preparations stained with toluidin blue exhibit a striking metachromasia of mucous alveoli. The latter stain a red-violet while the serous alveoli stain blue (orthochromatic).

Methyl Green Pyronin—Methyl green-pyronin stains mucous alveoli a green-blue. The serous alveoli stain pink-tan except for the nuclei which stain green.

Fig. 5.—Area of mucous gland treated with a phosphate buffer for fifteen minutes, showing partial solution of glycoprotein as fibrous strands (orig. mag. ×640).

Periodic Acid-Leukofuchsin Method—The mucous glands stain an intense red-violet (Fig. 2). Under higher magnification densely packed cytoplasmic aggregates of periodic acid-leukofuchsin positive granules are seen (Fig. 3). The serous alveoli stain less intensely (Fig. 2), and under higher magnification the granular appearance of the cytoplasm is more distinctly seen than that of the mucous cells (Fig. 4). Incubation of sections with either amylase or the control buffer alone prior to staining results in a similar diminution in stainability of both tissues. This indicates that appreciable amounts of glycogen were not present, and that the decrease in staining was caused by solution of water-soluble components.

Incubation with collagenase for one hour results in a very great diminution in stainability of the serous alveoli and to a lesser degree of the mucous alveoli. Buffer-treated sections also exhibit a diminished staining reaction, but to a lesser degree. In both enzyme and buffer-treated sections the remaining periodic acid-leukofuchsin positive material is diffusely distributed as compared with untreated sections.

Trypsin-incubated sections exhibit a diminution in the stainability of the mucous alveoli and an even greater diminution in that of the serous alveoli. Sections incubated in the buffer alone for five and fifteen minutes exhibited partial solution of material from the mucous alveoli. Periodic acid-leukofuchsin positive fibrous strands had obliterated the normal architecture of the gland (Fig. 5) (Table I).

TABLE I

STAINING REACTION OF CYTOPLASM OF MUCOUS AND SEROUS CELLS OF THE TONGUE

STAINING METHOD	MUCOUS CELLS	SEROUS CELLS
Mucicarmine	+	—
Toluidin blue	Metachromatic (red-violet)	Orthochromatic (blue)
Methyl green-pyronin	Methyl green	Pyronin (nuclei stain with methyl green)
Periodic acid-leukofuchsin	(Dark red-violet)	(Pink to red)

DISCUSSION

Metachromatic staining, differential staining with methyl green-pyronin, and the periodic acid-leukofuchsin reaction of salivary glands may be related to the state of organization of the intracellular precursors of mucous and serous secretions. Moreover, it is possible that differences in this organization may be reflected in gross physical charactistics of saliva, such as viscosity.

The metachromatic staining reaction of frozen dried tongue mucous glands confirms the work of Bensley[18] and others[4] who observed that mucous glands of the digestive tract as well as those of certain salivary glands exhibited this staining reaction. Subsequent studies have suggested that metachromatic staining may demonstrate biologic materials which are highly polymerized.[19, 20]

As previously indicated, the methyl green-pyronin stain has also been related to the state of organization of certain components of the tissue stained. Kurnick[7] claimed that desoxyribonucleic acid which is highly polymerized stains green, while the less polymerized ribonucleic acid stains red. Depolymerized desoxyribonucleic may also stain red. In this work the cytoplasmic contents of the mucous cells stain green, thus by analogy suggesting a different state of aggregation than the pyronin-staining cytoplasm of the serous cells.

With the periodic acid-leukofuchsin method the mucous alveoli stain most intensely and exhibit the greatest resistance to enzymatic breakdown and solution in buffers. The formation of fibrous strands as the contents of mucous alveoli go into solution suggests the polymerized nature of this substance, since many highly polymerized substances are known to form fibrous strands as they go into solution.[21]

CONCLUSIONS

The cytoplasm of the cells of mucous alveoli stains metachromatically with toluidin blue; blue-green, with methyl green-pyronin; and is intensely periodic acid-leukofuchsin positive.

The cytoplasm of serous alveoli stains orthochromatically with toluidin blue; pink-tan with methyl green-pyronin, and is periodic acid-leukofuchsin positive to a lesser degree than observed in the mucous alveoli.

The cytoplasmic contents of serous alveoli are more water soluble and more readily altered by enzymes than those of the mucous alveoli.

These observations, based upon histochemical procedures, suggest differences in the state of organization of glycoprotein components of mucous and serous salivary glands of the tongue.

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PIGMENT FORMATION IN THE RAT'S INCISOR

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DESPITE the numerous articles written on the rat's incisors, there seems to be no description of the origin of the pigment. The formation of the pigment has probably not been studied because the methods commonly employed in decalcifying the enamel destroy the pigment.

Shour and Smith[1] long ago observed that fluorine caused a reduction in the pigment of the exposed portion of the incisor. This diminution was explained on the basis that fluorine had a disturbing effect on the enamel epithelium in the middle portion of the tooth which is active in the formation of the pigment. Tempestini and Cannava[2] found that the pigment was retained and accentuated following microincineration.

Material recently prepared showed the formation of the pigment so well that the process seemed worth reporting, particularly since the incisors of the rat and their pigmentation serve as a basis for many studies.

EXPERIMENTAL PROCEDURE

Considerable advancement has been made in the preservation of the enamel matrix through the techniques developed by Sogannaes[3] and Villa.[4] Brain[5] has also contributed a procedure whereby much of the enamel matrix is retained after decalcification.

In the present study the mandibles from young rats were fixed in Bouin's or 10 per cent formalin. Each one was then put into a small tube with a small hole in the bottom and the tube was placed in a vial containing the solution developed by Sogannaes (100 c.c. 5 per cent trichloroacetic, 2.5 Gm. potassium bichromate, and 10 c.c. formalin). This solution was then changed daily and the vials kept in the refrigerator for three days. Following this the teeth were further decalcified in the same vials for two days in the solution recommended by Brain (5 per cent formic acid saturated with calcium phosphate). Both the enamel and dentin were better decalcified by this treatment than by either solution alone. The teeth in the tubes in the vials were then carefully washed in water and carried through increasing concentrations of ethyl to butyl alcohol. They were then removed to small dishes, infiltrated and embedded in paraffin. Sections stained in hematoxylin and slightly in eosin were as satisfactory as those in iron hematoxylin and Mallory's connective tissue stain.

To study the effects of fluorine on pigmentation of the incisors, 0.062 per cent sodium fluoride solution was given the rats as drinking water. When the exposed portion of the incisors showed no pigmentation, the animals were sacrificed. The teeth were then decalcified and prepared with the same procedure.

This investigation was supported by a research grant from the United States Public Health Service.

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OBSERVATIONS

A section of the normal incisor slightly distal to the level of the first molar or from Area III of Fig. 1 is shown in Fig. 2. Cell boundaries of the ameloblasts are not distinct in this photograph. Fine granular material is apparent in the ameloblasts. This is distinctly yellow in unstained sections. More apically at the level of the second molar, the yellowish granules are not found in the ameloblasts.

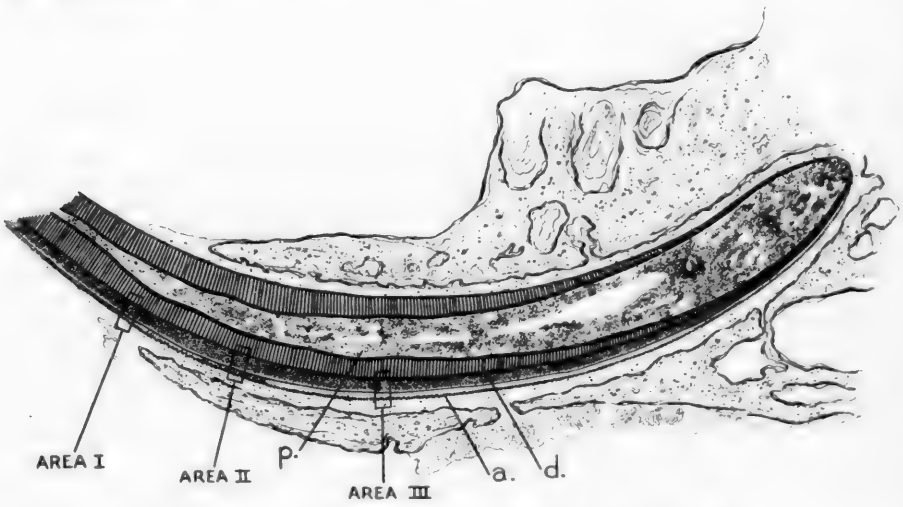
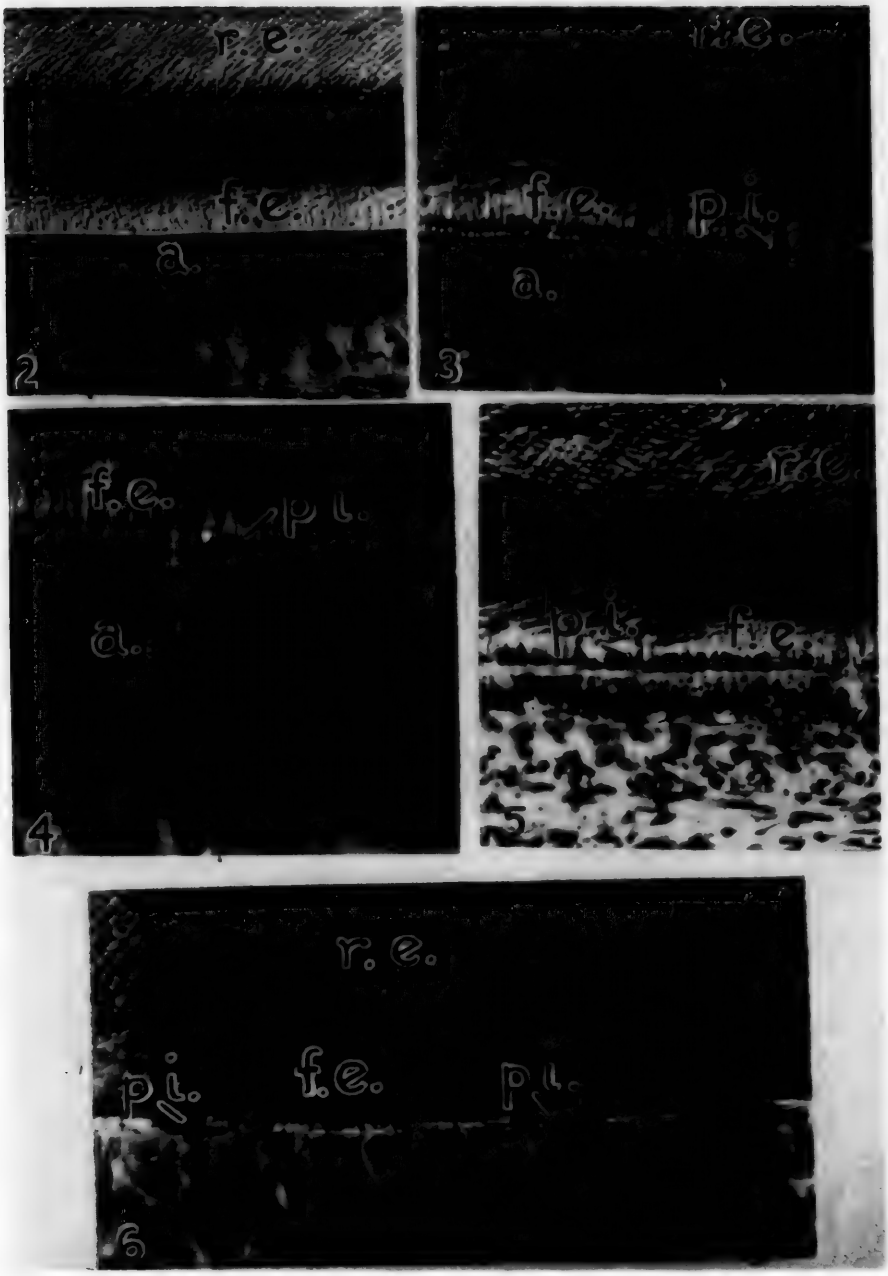


Fig. 1.—Drawing of incisor; *a.* ameloblast, *d.* dentin, *p.* pulp.

The structure of the enamel matrix in Area II of the incisor is seen in Fig. 3. The yellowish granular material of the ameloblasts has distally aggregated into globules of varying sizes. It is quite apparent that some of the yellowish pigment has passed into the outer fibrous enamel. A highly magnified section is shown in Fig. 4. Why the granular material accumulates into globules before deposition and how it enters the outer enamel is not quite clear. As a section of tooth is followed distally, however, the globular material in the ameloblasts is reduced and the amount of the pigment increased in the enamel, presenting evidence that the passage must occur.

A section from Area I (Fig. 5) shows that several changes have occurred. Most of the globules have disappeared from the ameloblasts and the pigment has greatly increased in the enamel. The height of the ameloblasts has been greatly reduced. The inner enamel, or about 60 per cent of the entire width of the enamel, shows distinct rods while the outer enamel appears to be fibrous in composition as described by Williams.[6] A limiting membrane or cuticle does not seem to be present. Cracks may be noted in the outer enamel indicating that it is considerably harder.

Distal to Area I of the incisor, remains of the ameloblasts, the stratum intermedium, and the papillary elevations gradually fuse into the enamel epithe-



Figs. 2 to 6.—Abbreviations: *a.* ameloblasts, *f.e.* fibrous enamel, *pi.* pigment, *r.e.* rod enamel.

Fig. 2.—Section of tooth from Area III of Fig. 1. (×270.)

Fig. 3.—Section from Area II, showing globules and pigment in fibrous enamel. (×450.)

Fig. 4.—Magnified section of Area II. (×700.)

Fig. 5.—Structures found in Area I. Few globules remain in the shortened ameloblasts. (×450.)

Fig. 6.—Section of tooth from rat given drinking water containing fluorine. (×550.)

lium. The oral epithelium appears to grow down between the enamel epithelium and the connective tissue as Skillen[7] showed it. At the gingival crest, however, most of the sections show a close fusion between the mouth epithelium and the enamel epithelium. Hornified cells at the gingival crest are carried out with the eruption of the tooth and they become the secondary cuticle.

There are two means by which the ingestion of fluorine may cause a reduction in the pigmentation. The ameloblasts may be affected and not produce the pigment. On the other hand, the pigment may be formed but not deposited in the enamel. The sections show that little pigment is formed. The small amount that does appear is confined to Area I where the ameloblasts are regressing. The pigment remains granular and fails to become globular. In many instances some pigment is retained by the ameloblasts as they fuse into the enamel epithelium. Occasionally clumps of pigment may be found between the enamel and the enamel epithelium (Fig. 6). Therefore, little pigment is formed and it fails to enter the enamel when fluorine is ingested.

DISCUSSION

According to Chase,[8] the later phases of maturation in the rat's incisor are completed before a point approximately one-third of the distance from the apical end to the gingival margin. Beyond this point all of the enamel is normally in the hard phase. Maturation is, therefore, apparently completed before the ameloblasts assume the function of producing pigment.

The most perplexing observation in this study is how the pigment passes from the cytoplasm of the ameloblasts into the fibrous outer enamel which is much denser and harder. However, since radioiodine (Bartelstone[9]) and other substances readily penetrate the enamel, the pigment may likewise possess this property. The pigment in these teeth appears to be quite similar to that which I have frequently observed[10] in their white hair.

In rats which received fluorine there is no difference in the thickness of the enamel nor in the proportions of rod and fibrous enamel. Pigment production is reduced and deposition of it in the enamel does not occur.

SUMMARY

Ameloblasts in the unexposed gingival portion of the rat's incisor produce granular pigment. As the tooth moves distally, these granules accumulate into globules which are deposited in the outer fibrous enamel.

In animals given fluorine, the ameloblasts begin the formation of the pigment late in their existence and little pigment is formed. Some pigment is retained by the ameloblasts while some is deposited between the enamel epithelium and the enamel.

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THE INHIBITION OF APPROXIMAL CARIES IN ADULTS WITH LIFELONG FLUORIDE EXPOSURE

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INDEPENDENT studies by Besic[1-3] and Nevin and Walsh[4] suggest that the diffusion of caries-inhibitory ions from the oral environment into the interproximal tooth area might be a function of the physical contours of the area itself, with the freest diffusion occurring in that approximal space with the least occluded area at the contact and with the widest embrasure. Nevin and Walsh consider this factor to be of greater importance than the buffering capacity of saliva. These studies imply that any caries-protective ion in saliva would act more effectively in the anterior than in posterior approximal areas.

Suess and Fosdick[5] reached a parallel conclusion. Since fluoridized enamel begins to decalcify at a point about 0.5 pH unit lower than nonfluoridized enamel, and since the surface pH of the anterior tooth is ordinarily higher than the pH of the posterior tooth which "is better protected from saliva," they suggest that "if the foregoing results and interpretations are correct, one would expect that the topical application of fluoride would provide almost complete protection to the anterior teeth and would provide less protection to the posterior teeth." They add: "Unfortunately, the clinical studies that have been performed to date do not provide data from this point of view."[5]

Raw data which could be analyzed from this standpoint were at hand in the dental examination records of Colorado adults with a lifelong exposure to a fluoride-bearing domestic water. The results of this analysis are, in general, consistent with the conclusions of all three studies cited.

MATERIAL AND METHOD

The populations from which these data are drawn and the procedures followed have been described in a previous report.[6] All were native adults with verified histories of birth, residence, and local water usage in Colorado Springs, Colorado, where the water contains about 2.5 parts per million of fluorides (F), or in Boulder, Colorado, where the water sporadically contains up to 0.1 part per million of fluorides (F) but is ordinarily fluoride-free. I made all of the examinations.

Studies of this type in children are complicated by biologic variation in the time-at-risk factor for individual tooth types. This difficulty is minimized when adults are considered. In older adults, however, excessive tooth loss is the rule and DMF tooth-surface findings are biased by the necessary inclusion of missing tooth surfaces which may not have been carious at the time of extraction. For these reasons the most desirable study group is the oldest in which tooth loss has been minimal. In the present populations there were more than eleven missing teeth per person in Boulder natives 35 years of age

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or older. Up to the age of 35 years, however, tooth mortality had been low in both cities, and adequate numbers for statistical validity were assembled by grouping persons aged 25 through 34 years in the two populations.

There were 68 such persons at Boulder* and 183 at Colorado Springs. Their mean ages were 29.0 and 29.6 years, respectively; tooth loss was 2.2 and 0.8 per person; DMF rates were 16.8 and 6.6 per person. The DMF frequency distributions were compact (with standard deviations of 5.3 and 4.9, respectively), a factor which considerably enhances the validity of these findings. With very few exceptions all of these people had received excellent and frequent dental care so that these findings include the minimum of tooth surfaces restored because a carious lesion, originating upon another surface, had been neglected until the approximal surface was involved.

The results of this analysis are shown in Table I.

TABLE I

PERCENTAGES OF PROXIMAL SURFACES AT RISK, BY APPROXIMAL AREAS, WHICH WERE DECAYED, OR MISSING, OR FILLED BECAUSE OF DENTAL CARIES, IN 68 BOULDER NATIVES AND 183 COLORADO SPRINGS NATIVES AGED 25-34 YEARS IN 1950

APPROXIMAL AREA	BOULDER			COLORADO SPRINGS			RATIO (PER CENT) DIFFERENCES TO BOULDER RATE
	NO. SURF.	NO. DMF	PER CENT DMF	NO. SURF.	NO. DMF	PER CENT DMF	
Maxillary Teeth							
Distals of second molars	136	49	36.0	363	50	13.8	-61.7
Distals of first molars and mesials of second molars	272	125	46.0	728	107	14.7	-68.0
Distals of second premolars and mesials of first molars	271	182	67.2	723	116	16.0	-76.2
Distals of first premolars and mesials of second premolars	270	156	57.8	721	79	11.0	-81.0
Distals of cuspids and mesials of first premolars	266	82	30.8	727	32	4.4	-85.7
Distals of second incisors and mesials of cuspids	259	54	20.8	715	17	2.4	-88.5
Distals of first incisors and mesials of second incisors	258	102	39.5	709	29	4.1	-89.6
Mesials of first incisors	130	42	32.3	358	17	4.7	-85.4
Mandibular Teeth							
Distals of second molars	136	60	44.1	365	40	11.0	-75.1
Distals of first molars and mesials of second molars	272	180	66.2	731	130	17.8	-73.1
Distals of second premolars and mesials of first molars	272	189	69.5	726	90	12.4	-82.2
Distals of first premolars and mesials of second premolars	272	88	32.4	724	28	3.9	-88.0
Distals of cuspids and mesials of first premolars	272	22	8.1	730	6	.82	-89.9
Distals of second incisors and mesials of cuspids	271	10	3.7	731	1	.14	-96.2
Distals of first incisors and mesials of second incisors	270	12	4.4	725	Zero	None	-100.0
Mesials of first incisors	135	6	4.4	360	Zero	None	-100.0

*Two Boulder natives aged 30 and 33 years who were wholly edentulous because of periodontal disease are omitted.

In order to test as directly as possible the hypotheses of Besic, and of Nevin and Walsh, approximal tooth areas are used as the unit of estimation in this analysis. This is a logical procedure in itself, in view of the known tendency for abutting tooth surfaces to become carious in pairs.[7] In Table I abutting tooth surfaces are considered to comprise a single approximal area; in the approximal area made up by the distal surface of the maxillary first incisor and the mesial surface of the second incisor, for example, about 40 per cent of all tooth surfaces at risk were decayed, missing, or filled because of dental caries in natives of Boulder and about 4 per cent at Colorado Springs, a decrease at Colorado Springs of about 90 per cent from the findings at Boulder. Each of the percentage values shown in Table I as ratios between rates at Colorado Springs and at Boulder has a standard error of approximately 4 percentage points* so that in general any two values which differ by more than 12 percentage points may be considered to be statistically different.

The relative susceptibility of the various approximal areas as seen in these populations is pictured in Fig. 1. In general, the patterns for the two groups are similar, with highest susceptibility in the region of the first molars and lowest susceptibility in the region of the lower anterior teeth. Susceptibility does not rise uniformly from the anterior to the posterior area of the mouth.

The apparent degree of caries inhibition following fluoride exposure, as measured by the differences between the two groups, is shown in Fig. 2. Inspection of this figure suggests that the caries-inhibiting effect of fluoride exposure diminishes in orderly fashion from the anterior to the posterior part of the mouth, independent of the fundamental susceptibility of the approximal area per se, and that mandibular teeth are somewhat better protected than their maxillary counterparts.

Since these factors show secondary relationships among themselves—there is, for example, a tendency for the more susceptible areas to occur toward the posterior part of the mouth—it is necessary to estimate the relationships between caries inhibition and susceptibility, anteroposterior position, and maxillomandibular position individually for each factor after it has been corrected for the influence of the other two. This was accomplished through the determination of partial regressions based upon relative rank, based upon rank values of 1 through 16 for susceptibility, values of 0 or 1 for position in the maxilla or mandible, and values of 1 through 8 for relative anteroposterior position in either jaw, with the results summarized in Table II.

This test indicates that, in these groups of people, anterior approximal areas were better protected against caries than were posterior approximal areas, and that mandibular approximal areas were better protected than maxillary approximal areas. Both patterns are independent of the basic susceptibility of the approximal area to dental caries.

*The values under "Per Cent DMF" in Table I were computed and analyzed as means, so that N = the number of persons examined. The use of proportion formulas in which N = the number of teeth is not appropriate in this situation.

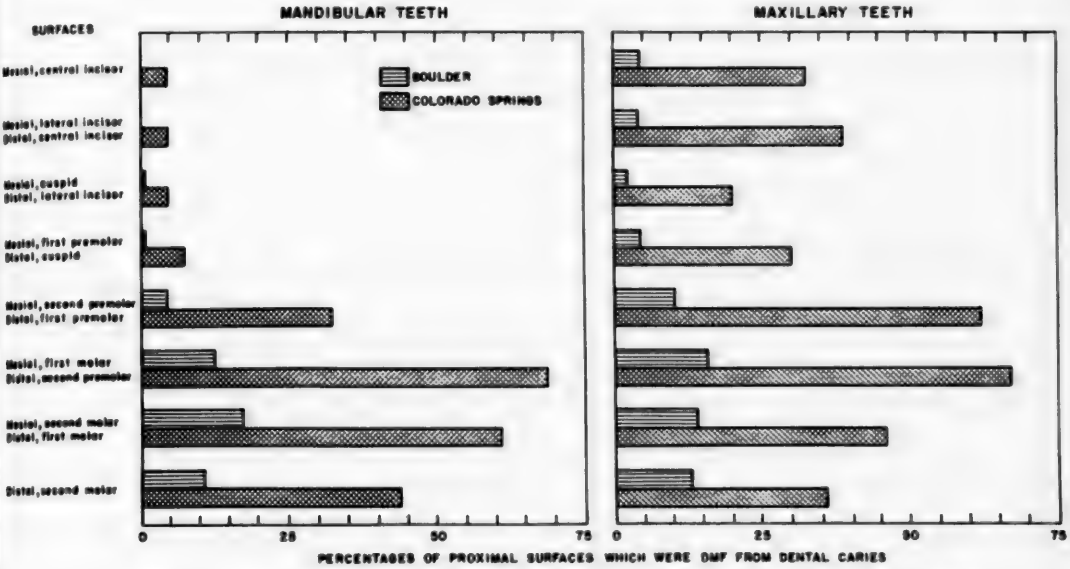

Fig. 1.—Percentages of approximal tooth surfaces which were decayed, missing, or filled because of dental caries in 68 natives of Boulder and in 183 natives of Colorado Springs, all aged 25 to 34 years.

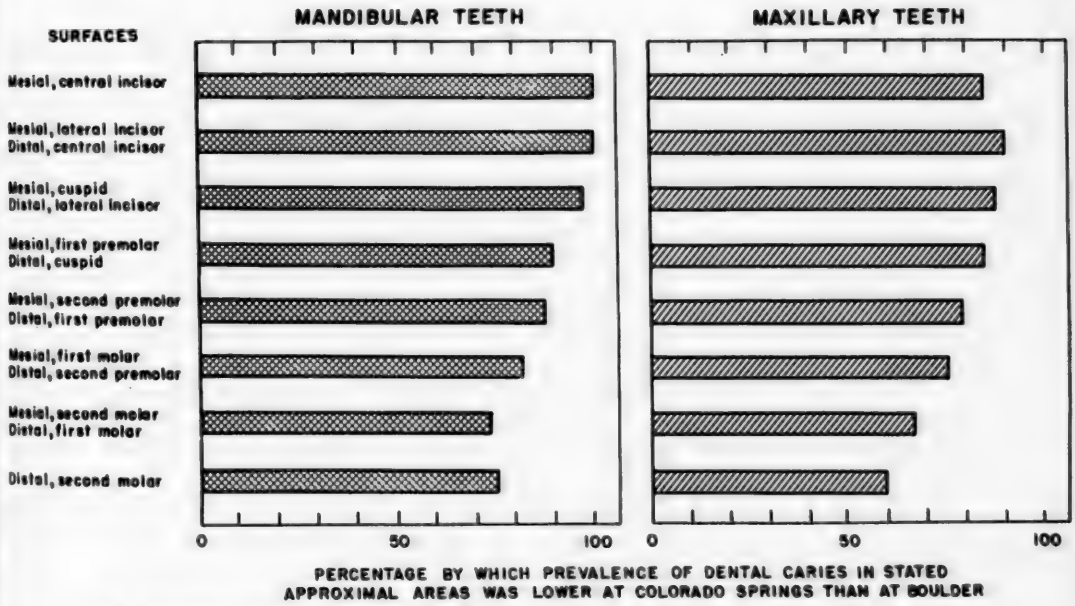

Fig. 2.—Apparent degree of inhibition by fluorides of dental caries on approximal tooth surfaces of 183 Colorado Springs natives aged 25 to 34 years.

TABLE II

PARTIAL REGRESSIONS FOR DEGREE OF CARIES INHIBITION FOLLOWING LIFELONG FLUORIDE EXPOSURE IN 183 NATIVES OF COLORADO SPRINGS, COLORADO, AGED 25-34 YEARS, AGAINST BASIC CARIES SUSCEPTIBILITY, ANTEROPOSTERIOR POSITION, AND MAXILLOMANDIBULAR POSITION OF APPROXIMAL TOOTH-SURFACE AREAS

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F	P
Multiple regression	298.18	3	99.39	28.9	<.001
Deviation from multiple regression (random variation)	41.32	12	3.44		
Partial regressions: degree of inhibition against					
Caries susceptibility	4.04	1	4.04	1.17	.30
Position, in maxilla or mandible	27.01	1	27.01	7.85	.02-.01
Position, posterior to anterior	89.31	1	89.31	25.94	<.001

DISCUSSION

These data apply only to smooth-surface approximal caries. Though the persons studied were adults 25 to 34 years of age with lifelong exposure to a fluoride water rather than children treated with topical fluorides as specified, the prediction of Suess and Fosdick that ". . . fluoride would provide almost complete protection to the anterior teeth and would provide less protection to the posterior teeth" is fully supported by these findings.

A possibility worthy of further investigation by physicochemical technics is that approximal contour affects not only the diffusion of buffering ions from saliva, but also as suggested by Besic, the ingress of the fluoride ion itself.[2] Caries inhibitions have been reported, in teeth fully calcified at the onset of fluoride exposure, in children relocated to a natural fluoride area,[8, 9] and in children using a fluoridated community water.[10] There is even some evidence that caries protection following fluoride exposure during the calcification period alone may be lost some years after a change to a fluoride-deficient water.[11] The need for further study in this area seems to be obvious.

SUMMARY

1. Patterns of dental caries inhibition in approximal tooth-surface areas, following lifetime fluoride exposure, were studied in 183 natives of Colorado Springs, Colorado, aged 25 to 34 years, with 68 comparable natives of Boulder, Colorado, as a control.

2. No direct relationship could be found between the basic caries-susceptibility of an approximal area and the degree of caries inhibition in that area. Instead there was a systematic diminution of caries inhibition from the anterior toward the posterior part of the mouth. Mandibular approximal surfaces were slightly better protected than were maxillary approximal surfaces.

The advice and assistance of Nathan Mantel, of the statistical laboratory of the National Cancer Institute, are gratefully acknowledged.

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Errata

In the August, 1952, issue of the JOURNAL, the article "Origin of Enamel Drops and Cementicles in the Teeth of Rodents" by V. Kalnins, p. 582, in the legend for Fig. 5*a*, (x) has been erroneously interchanged with (nr 5*a* is placed in this part). The description should read, "Appearance of angle between scorbutic (nr 5*a* is placed in this part) portions of molar of guinea pig and those developed during the cure of scurvy (x)." The legend for Fig. 16, ". . . . caused the folding of badly calcified dentin . . . ." should read instead ". . . . caused by folding of badly calcified dentin . . . ."

Also in the August issue, page 524, the name of *James Thewlis*, Atomic Energy Research Establishment, Harwell, England, was inadvertently omitted from the list of newly elected members.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Scientific Proceedings of the New York Section

New York University College of Dentistry—May 26, 1952

Compiled by Frances Krasnow, *Editor, New York Section*

1. A Clinical Evaluation of the High Potency Anesthetics—A Preliminary Report. *Harold Sherman, Joseph E. Fiasconaro, and Neal W. Chilton, School of Dental and Oral Surgery, Columbia University, New York, N. Y.* A blind clinical investigation was conducted on nonselected patients; 700 injections were administered by two operators in their offices. Seven local anesthetics were employed. Six were high-potency anesthetics of three manufacturers; 2 per cent procaine served as a standard. All solutions were placed in identical cartridges which were marked, packaged, and codified by the Cook-Waite Laboratories, Inc. The injection techniques were standardized as far as possible. All injections were timed. Thus 2.2 c.c. of each solution was administered over a 45 second period for the inferior alveolar block injection. Infiltrations were given within a 30 second period, utilizing 1.1 c.c. Operative work was started exactly five minutes after each injection. The sealed record of the code was kept in the Dean's office and was opened only after the following preliminary records were tabulated and released. The percentage figure represents the per cent of grade A anesthesia (complete elimination of pain) obtained for each for each solution. Novocain, Pontocain, Cobefrin—77 per cent; Win 3459-2 0.75 per cent with levophed 1:30,000—79 per cent; Xylocaine 2 per cent with epinephrin 1:50,000—84 per cent; Xylocaine 2 per cent with epinephrin 1:100,000—88 per cent; Monocaine 2 per cent with epinephrin 1:50,000—77 per cent; Unacaine 38 mg. per cubic centimeter with epinephrin 1:60,000—86 per cent; Procaine 2 per cent with epinephrin 1:50,000—61 per cent.

2. Enamel Lamellae and Their Origin. *Charles F. Bodecker, Dental Department, Montefiore Hospital, New York, N. Y.,* Abstracted in *J. D. Res.* **31**: 501, 1952.

3. A Method for In Vivo Recognition and Observation of Caries Lesions in the Molar Teeth of Syrian Hamsters. *Daniel A. Grant, Thomas J. Traynor, and Marvin Yarin,* Society of Dental Research, College of Dentistry, New York University, New York, N. Y.* The method proposed obviates some of the undesirable features of existing systems of caries recording in that it is more adaptable to different types of research and promises to give numerical results with greater reliability. Incidence and progression of carious lesions in the molars of Syrian hamsters are followed and charted by this method. Lateral jaw radiograms are taken at 1½ second exposure (radiatized film) on anesthetized animals. Films taken at regular intervals give the investigator serial progressions, wherein incidence and progression of the lesions may be followed and charted. Following sacrifice of the animals, the jaws are dissected and examined at 14 and 20 diameters magnification and lesions charted. The jaws are then x-rayed by direct technique and results correlated. Charting is adopted from the method of Keyes and can be adapted to serve the purpose.

4. Studies of the Biochemistry of Ossification in Relation to the Caries Problem.† *Albert Edward Sobel, Department of Biochemistry, Jewish*

*Guests.

†These studies were aided by a grant from the National Institute of Dental Research, National Institute of Health, United States Public Health Service.

Hospital of Brooklyn, Brooklyn, N. Y. The inorganic composition of bone, enamel, and dentin may be represented in the formula: $[Ca_3(PO_4)_2]_n \cdot CaCO_3$, where n equals 2.1 to 9.3. About 6 per cent of calcium may be replaced by magnesium, sodium, lead, etc. Some carbonate is probably replaced by citrate, fluoride, hydroxyl ion, sulfate, and other anions. Despite wide variation in inorganic composition, crystal structure is invariably apatite. After ignition at 900 degrees, structure changes to b-$Ca_3(PO_4)_2$ when the $Ca:PO_4$ mole ratio is 1.5 or less, and continues to be apatite when this ratio is 1.6 or more. Studies indicate that composition of bone and tooth mineral is related to composition of fluid from which precipitation takes place. When calcium acid phosphate is placed in a solution used for calcification in vitro, it changes to apatite (x-ray diffraction). $CO_3:PO_4$ ratio of this solid is related to $CO_3:PO_4$ content of solution. These experiments imply that if $CaHPO_4$ is first solid formed, as postulated, it can undergo change to apatite structure, and composition of new solid is related to composition of fluid. These experiments establish a relationship between in vivo and in vitro phenomena. The Ca_3PO_4 ratios, $Ca:PO_4$ ratio, and PO_4:citrate ratio of new mineral deposited during calcification in vitro are related to composition of fluid, and may be expressed as straight line plot, where logarithms of fluid ratios are plotted against mole ratios of solid. Similar relationship appears to exist between composition of blood serum and that of bone and teeth. $CO_3:PO_4$ ratio of growing enamel, dentin, and bone are related to serum $CO_3:PO_4$ ratios. Serum $CO_3:PO_4$ ratios are in turn related to dietary $Ca:PO_4$ ratios. Thus $CO_3:PO_4$ ratio of enamel, dentin, and bone can be influenced in predictable manner by regulating dietary Ca:P ratio. High calcium-low phosphorus diet produces high carbonate, whereas low calcium-high phosphorus diet produces low carbonate tooth and bone. As carbonate is preferentially dissolved from enamel, one would predict increased caries susceptibility with high carbonate enamel, compared to low carbonate enamel. That this is likely to be the case is indicated by experiments in cotton rat, where both incidence and degree of caries were about four times as high in animals having high carbonate tooth. Some of the factors that influence the site and degree of mineralization were intensively studied as continuation of earlier studies. In strontium or beryllium rickets, minimal product of calcium and phosphate ion required for calcification in vitro of rachitic bone cartilage is much higher than in the usual type of rickets, or in embryonic bone where minimal product is lowest. It is possible to reversibly inactivate mechanism responsible for calcification of preosseous cartilage matrix. Inactivation takes place by shaking with 150 mM/L of Ca^{++} in the presence of the following ions (inactivators): Be^{++} (0.1 mM/L), Cu^{++} (0.5 mM/L), Mg^{++} (10.0 mM/L), Na^{+} (25 mM/L), Sr^{+} (100 mM/L), K^{+} (400 mM/L). Reactivation takes place by shaking such sections with 150 mM/L of Ca. Inactivation is function of inactivator: Ca ratios. Results indicate combination with calcium ion is an essential preliminary step to mineralization. Fluoride, cyanide, and strontium ions inactivate or inhibit calcification in vitro in the presence of magnesium ions, whereas in the absence of magnesium ions, strontium ions are relatively mild inhibitors, cyanide is inactive, and fluoride enhances the degree of calcification. Further study of mechanism for reversible inactivation is of theoretical importance as a means of understanding the mechanism of calcification. On treating bones with various agents that inactivate calcifying mechanism, it was shown that following some types of treatment inactivation is reversible, while in others it is not. This may be regarded as destruction of calcifying mechanism. Special attention is called to the fact that after heating in water at 97° F. it was possible to reactivate calcifying mechanism. This treatment destroys enzymes of glycolytic cycle. In heating at same temperature with reactivating solution (0.15 M $CaCl_2$), it was not possible to reactivate calcifying mechanism; since $CaCl_2$ extracts chon-

droitin sulfate, whereas distilled water does not extract to the same degree, these experiments call attention to investigating role of chondroitin sulfate and related compounds in calcifying mechanism. Preliminary experiments with protamine as inactivator are in harmony with this concept.

5. Effect of Administration of Radioactive Iodine on Human Salivary Gland Function. *Leon H. Schneyer, Dental Department, Montefiore Hospital, New York, N. Y.* Preliminary investigation indicates that administration of I^{131} for treatment of thyroid carcinoma or hyperthyroidism also affects salivary gland function as measured by the level of salivary amylase. In three cases salivary amylase activity just prior to administration of an initial dose of activated iodine and at selected times after this for periods up to twenty-seven days was carefully determined. In each case, for as long as four days following administration, little or no effect on salivary amylase activity was noted. Following this period, the activity dropped markedly. Minimum activity was reached between the fourth and tenth days. Thereafter, amylase activity increased and returned to levels approximating the preadministration level. Amylase activity was measured by the Somogyi iodine method (*J. Biol. Chem.* **125**: 399, 1938). Mixed saliva, paraffin stimulated, was used diluted 1:2000 with distilled water. From the results obtained, it may tentatively be concluded that a single dose of activated iodine (8.-51. mc.) reversibly suppresses salivary gland function as measured by the level of the amylase activity.

6. A Biometric Study of Human Mandibular Segments. *Melvin L. Moss, Department of Anatomy, College of Physicians and Surgeons, New York, N. Y.* A method of measurement of the segments of human mandible is described and a statistical study of the variation exhibited. Segment isolation and essential asymmetry are demonstrated along with an evaluation of the factors producing over-all bilateral symmetry. There were 444 human mandibles measured. Three segments of the "neutral" portion of the mandible are located: from the caput of the condyle to the lingula of the mandibular foramen, from the latter to the mental foramen, and from the anterior border of this foramen to the symphysis menti. The constant asymmetry of each of these segments, the dextral dominance, and the failure of the mentongonion measurement to demonstrate these are statistically analyzed. The effect of endochondral and appositional bone growth gives a technic for ascertaining the quantitative aspects of each of these modes of bone growth and of the interaction between them. Some further applications of this technic for relative growth study and other physical anthropologic inquires are mentioned.

7. Modification of Skull and Jaw Architecture Following Removal of Masseter Muscle in the Rat. *Sidney L. Horowitz and Harry L. Shapiro, School of Dental and Oral Surgery and College of Physicians and Surgeons, New York, N. Y.* Abstracted in *J. D. Res.* **31**: 507, 1952.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Scientific Proceedings of the Philadelphia Section

Temple University, School of Dentistry—Nov. 17, 1952*

Compiled by Neal W. Chilton, *Editor, Philadelphia Section*

1. Blood Culture Techniques in Post-Extraction Bacteremias. *I. B. Bender, Albert Einstein Medical Center (Northern Division), Philadelphia, Pa.*

2. New Data on Caries Epidemiology, Treatment and Tooth Mortality From Dental Figures in School Health Examinations in Pennsylvania.

*Charles A. Wilde, Jr., presiding.

Benedict B. Kimmelman, Albert Einstein Medical Center, Philadelphia, Pa. Data published in the *Pennsylvania Dental Journal,* February, 1952, from type 3 standard dental examinations of 987,413 school children aged 6 to 18 years, during 1950 to 1951, were abstracted and presented as total DMF teeth, and as separate D, M, and F teeth per child at each age. DMF figures from this survey, increasing from 0.44 DMF teeth per child at the age of 6 years to 11.6 at the age of 18, were compared with composite figures from two groups of surveys, one conducted in 1933 to 1945, the other in 1947 to 1948. Comparison reveals that the early figures, which are still accepted source material, are appreciably below the latter figures and also below the Pennsylvania figures. This is taken as evidence of the inadequacy of earlier examinations and not as valid evidence of increase in caries experience. "D" figures in the Pennsylvania survey reveal unfilled needs ranging from 89 per cent at the age of 6 years to 60 per cent at the age of 18. "M" figures show the range of permanent tooth loss as 5 per cent of the dental needs at the age of 6 to 44 per cent at the age of 18, with one permanent tooth lost at the age of 13, another at 15, and a third at 18 years.

3. An Adhesive Dental Cement. *Fred A. Slack, Jr.,* University of Pennsylvania School of Dentistry, Philadelphia, Pa.* Newly extracted teeth were kept moist in normal saline solution until ground flat on the mesial or distal surface. These flats were then dried by air blast and covered with the adhesive cement being tested. Hardening occurred in the dry state and under water. The specimens were then tested by a smart rap with a child's hammer to demonstrate adhesiveness. The specimens were treated as follows: (1) freezing, (2) boiling, (3) drying, (4) wetting, (5) combination of other procedures. In all cases no demonstrable loosening of the bond was obtained after nine months. Similar specimens were cemented to the sides of glass jars. These jars were then rotated with flint stones inside to provide impact. Water was added to prevent heat and to dampen the effects, to simulate oral conditions. In all cases, the teeth fractured before the bond failed. It is conceded that these tests are quantitative only and the degree or rate of failure could not be ascertained. However, it is significant that all other cements could not even approach the results in these tests, and frequently failed before the test could be started.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Program of Minnesota Section

University of Minnesota, Dec. 4, 1952

AT THE regular fall meeting of the Minnesota Section held Thursday, Dec. 4, 1952, at the University of Minnesota, the following four papers were presented:

Dietary Magnesium Deficiency in the Hamster and Its Effects on the Periodontium: A Preliminary Report. Dr. George Yamane.

Electrophoresis in Dentin. Dr. Robert Sausen.

Investigations on the Occurrence and Significance of Citric Acid in the Saliva. Dr. Yngve Ericsson.

Thermogenesis During Cavity Preparation. Dr. William J. Simon.

*Guest.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Addendum to the Biographical Directory of Members

The names of a few members were omitted from the Biographical Directory of Members published in the October, 1952, issue of the JOURNAL. These include:

HILL, IDEN NAYLOR, 1806 Maple Ave., Evanston, Ill. *Dentistry.* Emporia, Kans., July 29, '11. D.D.S., Northwestern, '37. Assistant Zoller Dental Clinic, University of Chicago, '46-'48; Research Assoc. '48-'49; instructor, '49-'51; *Assoc. Professor,* '51-. Lieut. to Major, Dental Corps, U.S.A.F., '42-'45. Dental Surgeon, Chicago Board of Health, '38-'42; A.D.A., 111 S.D.A. Chic. D.S. Dental caries; fluorine.

KLATSKY, MEYER, 401 Broadway, New York 13, N. Y. D.D.S., *Dentistry,* New York College of Dentistry, '15. A.D.A. Physiology of mastication; caries and mastication attrition.

MOORE, ALTON W., Dental School, University of Washington, Seattle, Wash. *Orthodontics.* D.D.S., Illinois, '41, M.S., '45. Private pract., Chicago, '46-'48. *Professor of orthodontics,* University of Washington. A.D.A., A.A.O., facial growth.